THE NIH LIBRARY
NATIONAL INSTITUTES OF HEALTH

THE NIH LIBRARY
NATIONAL INSTITUTES OF HEALTH

NCI Monographs

A Publication
of the National
Cancer Institute

Development of Folates and Folic Acid Antagonists in Cancer Chemotherapy

1987
Number 5

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service National Institutes of Health

NCI Monographs

National Cancer Institute, Vincent T. DeVita, Jr., ***Director***
International Cancer Information Center, Susan Molloy Hubbard, ***Director***

Editorial Board

Robert E. Wittes, ***Editor-in-Chief***

Associate Editors

Joseph Aisner
Lewis Aronow
Gregory Curt
Richard L. Cysyk
Ross C. Donehower
Michael Friedman
Timothy J. Kinsella
Robert F. Ozols
Carl Pinsky
David G. Poplack
Richard Simon

Advisory Board

Charles M. Balch
Robert C. Bast, Jr.
Gianni Bonadonna
Edwin C. Cadman
Giulio J. D'Angio
I. David Goldman
Heine H. Hansen
Simon Kramer
Susan E. Krown
John E. Niederhuber
T. L. Phillips
H. M. Pinedo
Carol S. Portlock
Joseph V. Simone
Samuel A. Wells, Jr.

Publications Branch, Robin A. Atkiss, *Chief*

Although the National Cancer Institute was a co-sponsor of the conference, editorial services for this monograph were supplied by Park Row Publishers, New York, NY, under a nonfederal contract.

Editorial Policy

Manuscripts from key conferences dealing with cancer and closely related research fields, or a related group of papers on specific subjects of importance to cancer research, are considered for publication, with the understanding that they have not been published previously and are submitted exclusively to *NCI Monographs.* All material submitted for consideration will be subject to review, when appropriate, by at least two outside reviewers and one member of the *JNCI* or *Cancer Treatment Reports* Editorial Board. Opinions expressed by the authors are not necessarily those of the publisher or the editors.

Symposia or related papers in any of the following areas should be submitted to the Editor-in-Chief, *Cancer Treatment Reports:* surgery, radiotherapy, chemotherapy, biologic response modification, supportive care, pharmacology, mechanisms of drug action, and medicinal chemistry. Symposia or related papers in cancer etiology, molecular biology, prevention, control, or other areas of cancer research should be submitted to the Editor-in-Chief, *JNCI.*

The editorial offices for both journals are located at the R. A. Bloch International Cancer Information Center, Building 82, Room 209, National Cancer Institute, Bethesda, MD 20892.

Development of Folates and Folic Acid Antagonists in Cancer Chemotherapy

Innisbrook
Tarpon Springs, Florida
January 23-26, 1986

Symposium Chairman:

Peter J. O'Dwyer

Sponsors:

National Cancer Institute
Burroughs Wellcome Co.
Lederle Laboratories

Session Moderators:

Howard Bruckner
Bruce A. Chabner
Daniel Hoth
Bernard Levin
Robert E. Wittes

TABLE OF CONTENTS

Page

Folic Acid Metabolism and Its Disruption by Pharmacologic Agents

F. M. Huennekens, T. H. Duffy, and K. S. Vitols 1

Unresolved Issues in the Biochemical Pharmacology of Antifolates

Robert C. Jackson 9

Biochemical Factors in the Selectivity of Leucovorin Rescue: Selective Inhibition of Leucovorin Reactivation of Dihydrofolate Reductase and Leucovorin Utilization in Purine and Pyrimidine Biosynthesis by Methotrexate and Dihydrofolate Polyglutamates

I. David Goldman and Larry H. Matherly 17

Determinants of Resistance to Antifolates: Biochemical Phenotypes, Their Frequency of Occurrence and Circumvention

F. M. Sirotnak 27

Folate and Antifolate Cell Binding and Accumulation at Physiologic Folate Concentrations

Barton A. Kamen 37

Pharmacokinetics of Leucovorin (D,L-5-Formyltetrahydrofolate) After Intravenous Injection and Constant Intravenous Infusion

J. A. Straw, E. M. Newman, and J. H. Doroshow 41

Absorption Kinetics of Orally Administered Leucovorin Calcium

Brian W. McGuire, Luisa L. Sia, John D. Haynes, James C. Kisicki, Manuel L. Gutierrez, and E. L. Robert Stokstad 47

Bioavailability of High-Dose Oral Leucovorin

John D. Hines, Michael H. Zakem, David J. Adelstein, Paul Giroski, M. Robert Blum, and Youcef M. Rustum 57

Biochemical and Pharmacologic Rationale for High-Dose Methotrexate

Jacques Jolivet 61

High-Dose Methotrexate in Osteosarcoma—Mayo Clinic Studies

John H. Edmonson 67

Methotrexate-Leucovorin Factor Rescue Regimens in Diffuse Large Cell Lymphoma

Arthur T. Skarin and George P. Canellos 71

A Randomized Trial of High-Dose Methotrexate Versus Standard-Dose Methotrexate Following Cyclophosphamide, Doxorubicin (Adriamycin), Vincristine, and Prednisone With or Without Bleomycin in the Therapy of Diffuse Large Cell Lymphoma: Preliminary Report of Cancer and Leukemia Group B Study 7851

Sandra J. Ginsberg, James R. Anderson, Arlan J. Gottlieb, Clara D. Bloomfield, Larry Norton, Maurice Barcos, and James F. Holland 77

Clinical Pharmacodynamic Studies of High-Dose Methotrexate in Acute Lymphocytic Leukemia

William E. Evans, Minnie Abromowitch, William R. Crom, Mary V. Relling, W. Paul Bowman, Ching-Hon Pui, Judith Ochs, and Richard Dodge 81

Design and Rationale for Novel Antifolates

J. R. Bertino, A. Sobrero, E. Mini, B. A. Moroson, and A. Cashmore 87

Methodology for the Rational Development of Methotrexate Analogs in the Clinic

Brian Leyland-Jones, Peter J. O'Dwyer, Daniel F. Hoth, and Robert E. Wittes 93

Therapeutic Synergy of Trimetrexate (CI-898) in Combination With Doxorubicin, Vincristine, Cytoxan, 6-Thioguanine, Cisplatin, or 5-Fluorouracil Against Intraperitoneally Implanted P388 Leukemia

Wilbur R. Leopold, Donald J. Dykes, and Daniel P. Griswold, Jr. 99

Trimetrexate: Clinical Development of a Nonclassical Antifolate

Peter J. O'Dwyer, Robert J. DeLap, Susan A. King, A. J. Grillo-Lopez, Daniel F. Hoth, and Brian Leyland-Jones 105

Preclinical Biochemical Pharmacology and Toxicology of Piritrexim, a Lipophilic Inhibitor of Dihydrofolate Reductase

C. W. Sigel, A. W. Macklin, J. L. Woolley, Jr., N. W. Johnson, M. A. Collier, M. R. Blum, N. J. Clendeninn, B. J. M. Everitt, G. Grebe, A. Mackars, R. G. Foss, D. S. Duch, S. W. Bowers, and C. A. Nichol 111

Initial Clinical Studies of Piritrexim

J. Laszlo, W. D. Brenckman, Jr., E. Morgan, Neil J. Clendeninn, T. Williams, V. Currie, and C. Young 121

10-Ethyl-10-Deaza-aminopterin: Structural Design and Biochemical, Pharmacologic, and Antitumor Properties

F. M. Sirotnak, F. A. Schmid, L. L. Samuels, and J. I. DeGraw 127

Structural Requirements for the Activity of Antifolates as Substrates for Mammalian Folylpolyglutamate Synthetase

Richard G. Moran, Paul D. Colman, and Andre Rosowsky 133

Folate Analog Nonsubstrates and Inhibitors of Folylpolyglutamate Synthetase as Potential Cancer Chemotherapy Drugs

John J. McGuire, James R. Piper, James K. Coward, and John Galivan 139

Synthesis and Biologic Activity of New Side-Chain-Altered Methotrexate and Aminopterin Analogs With Dual Inhibitory Action Against Dihydrofolate Reductase and Folylpolyglutamate Synthetase

Andre Rosowsky, Richard G. Moran, James H. Freisheim, Henry Bader, Ronald A. Forsch, and Vishnu C. Solan 145

Antifolate Metabolism and Sites of Action: Implications for the Design of New Antifolates

Anthony L. Fitzhugh, Bruce A. Chabner, Pam W. Sholar, Jacob Baram, and Carmen J. Allegra 153

Biochemical Rationale for the Synergism of 5-Fluorouracil and Folinic Acid

Richard G. Moran and Khandan Keyomarsi 159

Biochemical and Pharmacologic Basis for Potentiation of 5-Fluorouracil Action by Leucovorin

Y. M. Rustum, F. Trave, S. F. Zakrzewski, N. Petrelli, L. Herrera, A. Mittelman, S. G. Arbuck, and P. J. Creaven 165

Preliminary Analysis of a Randomized Comparison of 5-Fluorouracil Versus 5-Fluorouracil and High-Dose Continuous-Infusion Folinic Acid in Disseminated Colorectal Cancer

James H. Doroshow, Marcelle Bertrand, Edward Newman, Phyllis Multhauf, Lucille Leong, Douglas Blayney, David Goldberg, Kim Margolin, Brian Carr, Stephen Akman, and Gerald Metter 171

A Northern California Oncology Group Randomized Trial of Leucovorin Plus 5-Fluorouracil Versus Sequential Methotrexate, 5-Fluorouracil, and Leucovorin in Patients With Advanced Colorectal Cancer Who Failed Treatment With 5-Fluorouracil or 5-Fluorodeoxyuridine Alone

Frank H. Valone, Marsha Kohler, Karen Fisher, John Hannigan, Marshall Flam, David Gandara, Charles Hendrickson, Eli Richman, and Kou-Ping Yu 175

Comparison of Unique Leucovorin and 5-Fluorouracil "Escalating" and "Maximum" Dosage Strategies

Howard W. Bruckner, Nicholas J. Petrelli, Donald Stablein, Joel Novak, and Robert Mayer 179

Clinical Studies of Biochemical Modulation of 5-Fluorouracil by Leucovorin in Patients With Advanced Colorectal Cancer by the North Central Cancer Treatment Group and Mayo Clinic

Michael J. O'Connell, David J. Klaassen, Lloyd K. Everson, Stephen Cullinan, and Harry S. Wieand 185

Biologic Modulation of 5-Fluorouracil With High-Dose Leucovorin and Combination Chemotherapy of 5-Fluorouracil and Cisplatin in Metastatic Colorectal Adenocarcinoma

Nicholas J. Petrelli, Stefan Madajewicz, Lemuel Herrera, Youcef M. Rustum, Fabio Trave, Patrick Creaven, and Arnold Mittelman 189

Treatment of Advanced Colorectal and Gastric Adenocarcinomas With 5-Fluorouracil and High-Dose Folinic Acid

David Machover, Emma Goldschmidt, Philippe Chollet, Gérard Metzger, Jacqueline Zittoun, Manuel Benavides, Jeanine Marquet, Jean-Marie Vandenbulcke, Jean-Louis Misset, Léon Schwarzenberg, Jean-Pierre Lotz, Jean B. Fourtillan, Hélène Gaget, and Georges Mathé 193

Preliminary Results of a Phase II Trial for the Treatment of Metastatic Breast Cancer With 5-Fluorouracil and Leucovorin

Carmen J. Allegra, Bruce A. Chabner, Pam W. Sholar, Caroline Bagley, James C. Drake, and Marc E. Lippman 199

A Phase II Trial of 5-Fluorouracil and High-Dose Leucovorin in Gastric Carcinoma and a Phase I Trial of Intraperitoneal 5-Fluorouracil and Leucovorin

Susan G. Arbuck, Harold O. Douglass, Jr., Fabio Trave, and Youcef M. Rustum 203

Transient Acute Hepatotoxicity of High-Dose Methotrexate Therapy During Childhood

Barbara L. Weber, Gulten Tanyer, David G. Poplack, Gregory H. Reaman, James H. Feusner, James S. Miser, and W. Archie Bleyer 207

Recent Preclinical and Clinical Studies With the Thymidylate Synthase Inhibitor N^{10}-Propargyl-5,8-dideazafolic Acid (CB 3717)

A. Hilary Calvert, David R. Newell, Ann L. Jackman, Lindsey A. Gumbrell, Ewa Sikora, Barbara Grzelakowska-Sztabert, Joel A. M. Bishop, Ian R. Judson, Stephen J. Harland, and Kenneth R. Harrap 213

Cisplatin and Dichloromethotrexate: A Pharmacologically Rational Combination

Ronald B. Natale, Richard H. Wheeler, and James A. Roberts 219

Folic Acid Metabolism and Its Disruption by Pharmacologic Agents[1-3]

F. M. Huennekens,[4,*] T. H. Duffy,[4,5] and K. S. Vitols[4]

ABSTRACT—Folic acid metabolism in eukaryotic cells consists of a network of enzymatic reactions in which 1-carbon (C_1) units at three different oxidation states are 1) interconverted while linked to the 5- and/or 10-positions of tetrahydrofolate, or 2) added to, or taken from, tetrahydrofolate. Particularly important in the latter category are reactions involving C_1-tetrahydrofolate adducts in the synthesis of inosinate, thymidylate, serine, and methionine. Tetrahydrofolate, a central component of the network, can be generated from: 1) folate, via the NADPH-dependent dihydrofolate reductase; 2) 5-methyltetrahydrofolate via the methyl B_{12}-dependent methionine synthetase; or 3) 5-formyltetrahydrofolate via a sequence of reactions beginning with the ATP-dependent isomerization to 5,10-methenyltetrahydrofolate or via transfer of the formyl group to glutamate. Because of the close relationship of folic acid metabolism to cell replication, folate-dependent enzymes provide excellent targets for cancer chemotherapy. This potential has not yet been realized, however, except for dihydrofolate reductase and thymidylate synthetase, which are strongly inhibited by the anti-cancer agents methotrexate (MTX) and FUra. The following enzymes are particularly attractive as targets for future exploitation in chemotherapy: 1) the two transformylases involved in purine nucleotide synthesis, 2) serine hydroxymethyltransferase, 3) methionine synthetase, and 4) methylenetetrahydrofolate dehydrogenase. Suggestions are also made for the development of new agents based upon a strategy of enzyme-targeted chemotherapy—NCI Monogr 5:1-8, 1987.

Folate-dependent enzymes, which are subsumed within the general category of "folic acid metabolism," occur universally in eukaryotic cells. Although all areas of metabolism contribute to the overall economy of the cell, some are more important than others. [This is reminiscent of George Orwell's definition of a democracy: "All animals are equal, but some are more equal than others." (*1*)] Folic acid metabolism is especially important because of its crucial role in cell replication. Folate-dependent enzymes, therefore, represent prime targets for cancer chemotherapeutic agents.

Eukaryotes, unable to synthesize folate compounds, require an external source of these materials and a mechanism for their internalization. The latter requirement is met by an active transport system that is concentrative, energy-dependent, and carrier-mediated [reviewed in (*2-5*)]. Anion gradients provide the energy source (*5*), and the carrier is a membrane-associated protein. Folate transport is closely linked to intracellular folate metabolism (fig. 1). After being internalized, folates are converted to polyglutamates; (this conversion ensures intracellular retention and generally increases their affinity for enzymes). They are also derivatized to form various C_1 adducts. It is not yet clear, however, whether polyglutamylation precedes, follows, or occurs concomitantly with adduct formation. Both transport and polyglutamylation are excellent, although still largely unrealized, targets for cancer chemotherapy. Even more attractive is the array of reactions involved in the production, interconversion, and utilization of the C_1-folate adducts, particularly since 2 of the drugs in current use, MTX and FUra, owe their efficacy to inhibition of enzymes that catalyze reactions in this metabolic area.

Folate metabolism consists of a set of enzymatic reactions in which the folate coenzyme, tetrahydrofolate (fig. 2), serves as the central component. As illustrated by the horizontal hierarchies in figure 3, this compound provides the scaffolding for C_1 units at three different oxidation states (formate, formaldehyde, and methanol); these may be attached to the 5- or 10-positions (or both) of tetrahydrofolate. Also shown is the interrelationship among five of the known C_1-tetrahydrofolate adducts [5-formyltetrahydrofolate (5-CHO-FH_4), 5,10-methenyltetrahydrofolate (5,10-CH-FH_4), 10-formyltetrahydrofolate (10-CHO-FH_4), 5,10-methylenetetrahydrofolate (5,10-CH_2-FH_4), and 5-methyltetrahydrolate (5-CH_3-FH_4)]. 5-Formiminotetrahydrofolate also occurs in cells, but it is omitted in the figure for lack of relevance to the present discussion. Interconversion of the C_1 units can be achieved while they are linked to tetrahydrofolate; for example, 5,10-CH-FH_4 and 5,10-CH_2-FH_4 are connected reversibly via a pyridine nucleotide-

---

ABBREVIATIONS: AICAR = 5-amino-4-imidazolecarboxamide ribonucleotide; C_1 = 1-carbon; 5,10-CH-FH_4 = 5,10-methenyltetrahydrofolate; 5,10-CH_2-FH_4 = 5,10-methylenetetrahydrofolate; 5-CH_3-FH_4 = 5-methyltetrahydrofolate; CHO-FH_4 = formyltetrahydrofolate; GAR = glycinamide ribonucleotide; ID_{50} = concentrations required for 50% inhibition of growth; MTX = methotrexate; P_i = inorganic phosphate; SAM = *S*-adenosylmethionine; TC II = transcobalamin II.

[1] Supported by an Outstanding Investigator grant from the National Cancer Institute, National Institutes of Health (CA-39636) and a grant from the American Cancer Society (CH-31).

[2] This is publication number 4257-BCR from the Research Institute of Scripps Clinic.

[3] We thank Drs. Laura Pope, Gerhard Grundler, and Judith Sato for discussions on folate antagonists as chemotherapeutic agents; Yolanda Montejano for technical assistance; and Susan Burke for typing the manuscript.

[4] Division of Biochemistry, Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, CA.

[5] T. H. Duffy is the recipient of a Postdoctoral Fellowship (CA-07660) from the National Institutes of Health.

* *Reprint requests to:* F. M. Huennekens, Ph.D., Division of Biochemistry, Department of Basic and Clinical Research, BCR-7, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037.

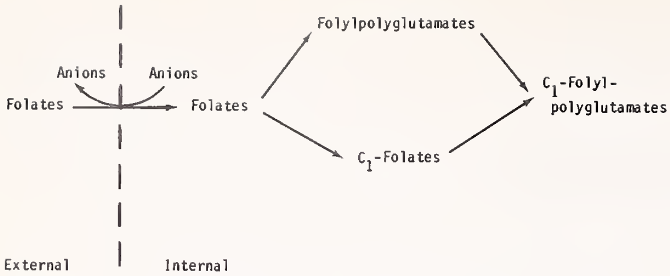


FIGURE 1.—Folate compounds: Transport, polyglutamylation, and conversion to C_1 adducts.

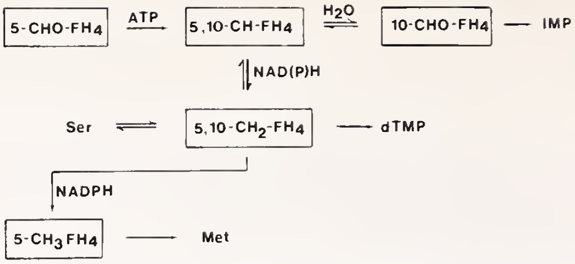


FIGURE 3.—Role of folate coenzyme, tetrahydrofolate, in synthesis of inosinate (IMP), serine (Ser), thymidylate (dTMP), and methionine (Met).

dependent enzyme, but the NADPH-dependent reduction of 5,10-CH_2-FH_4 to the 5-methyl derivative is irreversible. Involvement of the C_1-tetrahydrofolate adducts in the synthesis of inosinate, thymidylate, serine, and methionine is also illustrated in figure 3. Many versions of this diagram exist; the one shown here is an abbreviated version of the prototype first introduced in 1958 (*6*). Omitted from figure 3 are a few folate-dependent reactions that occur in eukaryotic cells but are not relevant to the present discussion (e.g., degradation of histidine via formiminoglutamate) and reactions that are found only in bacteria. Likewise, reactions that utilize pterins rather than folates (e.g., hydroxylation of phenylalanine) are not included. Even with these limitations, a considerable amount of complicated chemistry is represented by the reactions in figure 3. It seems likely that much trial and error must have occurred during evolution before a single compound, tetrahydrofolate, was found to possess the structural features necessary for these diverse reactions.

The primary purpose of the network is the production of purine and pyrimidine precursors for DNA and RNA. This is accomplished by utilization of 10-formyltetrahydrofolate to supply the C_1 unit for the 2- and 8-positions of inosine monophosphate (the precursor of both AMP and GMP) and of 5,10-CH_2-FH_4 for the 5-methyl group of thymidylate. Serine can also be synthesized via a folate-dependent enzyme, and the reaction is one of the few in this network that is reversible. It is likely, however, that the reaction normally proceeds in the opposite direction with serine (arising from glucose) serving to funnel C_1 units into the system. Similarly, the synthesis of methionine from 5-CH_3-FH_4 is generally insufficient to meet the cellular need for this essential amino acid, and thus the primary function of the reaction would appear to be the generation of tetrahydrofolate from a more stable precursor.

In all of the reactions discussed above, except for thymidylate synthesis, the oxidation state of the coenzyme remains unchanged regardless of the fate of the C_1 unit. In the synthesis of thymidylate, however, transfer of the C_1 group is accompanied by its reduction from the methylene to the methyl level. The latter step is coupled to an oxidation in the pyrazine ring of tetrahydrofolate to yield 7,8-dihydrofolate. Regeneration of 5,10-CH_2-FH_4 then requires the sequential action of dihydrofolate reductase and serine hydroxymethyltransferase (fig. 4). The "thymidylate synthesis cycle" is discussed below.

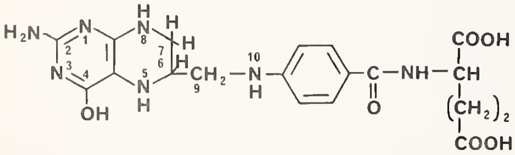


FIGURE 2.—Structure of tetrahydrofolate.

The route for production of tetrahydrofolate depends upon the folate compound to which cells are exposed (fig. 5). In the body, cells are nurtured primarily by 5-CH_3-FH_4, but in the laboratory they are propagated on folate (or occasionally on 5-CH_3-FH_4 or 5-CHO-FH_4). 5-CH_3-FH_4 is demethylated to the coenzyme form by the B_{12}-dependent methionine synthetase, whereas folate is reduced to tetrahydrofolate via the NADPH-dependent dihydrofolate reductase. 5-CHO-FH_4 (folinate) is converted to the coenzyme either by means of an indirect process, the first step of which is the ATP-dependent transformation to 5,10-CH-FH_4 (fig. 3), or by direct transfer of the formyl group to glutamate (not shown).

Figures 4 and 5 help explain the complexity of MTX cytotoxicity. Regardless of the folate substrate used to propagate the cells, MTX is cytotoxic because it inhibits the reduction of dihydrofolate in the thymidylate synthesis cycle. In principle, cells given very high levels of 5-CH_3-FH_4 or 5-CHO-FH_4 (but not folate) should be able to replicate in the presence of MTX, but this is difficult to achieve experimentally. But because it also inhibits the reduction of folate in the de novo generation of tetrahydrofolate, MTX will be even more cytotoxic when cells are grown on folate. Thus, when cell cultures are used for screening MTX or its analogs, the most relevant concentrations required for 50% inhibition of growth (ID_{50}) from a physiological viewpoint will be obtained when 5-CH_3-FH_4 is used in the growth medium.

The network in figure 3 operates like a set of gear wheels. Inhibitors that affect any one of the reactions would be expected to produce changes in the others. In turn, these

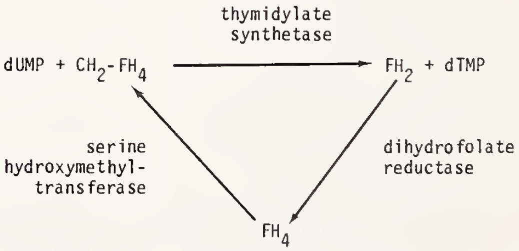


FIGURE 4.—Thymidylate synthesis cycle. FH_2=dihydrofolate; FH_4=tetrahydrofolate.

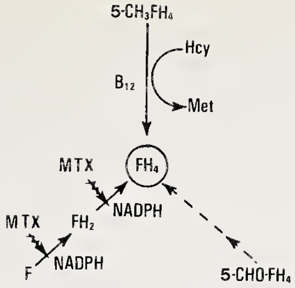


FIGURE 5.—Routes for production of tetrahydrofolates. F=folate; Hcy=homocysteine. Inhibition indicated by ⇸

changes would alter the amounts of the various C_1-tetrahydrofolate adducts. The latter can be determined experimentally by high-performance liquid chromatography (*7*), and the results should correlate with the activities of the enzymes that control the production and utilization of these compounds. However, apparent "levels" of these enzymes, measured in cell-free extracts and using optimal assay conditions, may not correspond to their activities in vivo. Nevertheless, even though limited by the fragmentary data available, Jackson and Harrap (*8*) were able to employ computer simulation for analysis of a network similar to figure 3 (but extending to the nucleotide precursors of DNA and RNA) and for predicting the effects of drugs targeted to specific enzymes.

FOLATE METABOLISM: SPECIFIC STEPS

Oxido-reduction of C_1 Units Bound to Tetrahydrofolate

The 5,10-methenyl and 5,10-methylene derivatives of tetrahydrofolate are interconvertible via a reversible reaction mediated by pyridine nucleotide-dependent dehydrogenases:

$$\text{5,10-CH-FH}_4+\text{NAD(P)H} \rightleftharpoons \text{5,10-CH}_2\text{-FH}_4+\text{NAD(P)} \quad \text{(eq. 1)}$$

Mackenzie (*9*) has recently made the interesting observation that tumor cells appear to use an NAD-dependent enzyme for this purpose, while in normal cells an NADP-dependent enzyme is operative. This unique difference, coupled with the central position of $\text{5,10-CH}_2\text{-FH}_4$ in the metabolic network, makes the tumor enzyme an attractive target for chemotherapeutic agents. As yet, however, no systematic search has been made for inhibitors of this enzyme.

Reduction of $\text{5,10-CH}_2\text{-FH}_4$ to the 5-methyl derivative is an irreversible reaction, catalyzed by an NADPH-dependent enzyme (*10*):

$$\text{5,10-CH}_2\text{-FH}_4+\text{NADPH} \rightarrow \text{5-CH}_3\text{-FH}_4+\text{NADP} \quad \text{(eq. 2)}$$

The product $\text{5-CH}_3\text{-FH}_4$ thus lies in a trap, from which the only escape is via the reaction catalyzed by methionine synthetase (discussed below). The purpose of the above reaction is not clear. It may provide: 1) a stable storage form for reduced folates, which could be activated, as needed, by the methionine synthetase-catalyzed reaction; 2) a mechanism for suppressing the synthesis of purines and thymidylate by sequestering reduced folates; or 3) a route for the production of methionine when the latter, perhaps unavailable from external sources, is needed for special purposes. Methylenetetrahydrofolate reductase is inhibited by *S*-adenosylmethionine (SAM), and this is believed to be a regulatory mechanism for turning off production of methionine. Since SAM is a weak, allosteric inhibitor [50% inhibition of the pig liver enzyme at approximately 100 μM (*11*)], and since charged compounds of this type are not internalized readily, it seems unlikely that SAM analogs will be useful in chemotherapy. A more impressive inhibition of the enzyme is obtained with the hexaglutamate of dihydrofolate [inhibitory constant $(K_i)=0.01\ \mu M$, which may be compared to the Michaelis constant (K_m) of 0.1 μM for the hexaglutamate of the substrate, $\text{5,10-CH}_2\text{-FH}_4$ (*10*)]

Interconversion of Formyltetrahydrofolates

5-CHO-FH_4 can be converted to 5,10-CH-FH_4 by an ATP-dependent enzyme (5,10-CH-FH_4 synthetase), but the reaction appears to be irreversible (*12*):

$$\text{5-CHO-FH}_4+\text{ATP} \rightarrow \text{5,10-CH-FH}_4+\text{ADP}+\text{P}_i \quad \text{(eq. 3)}$$

where P_i = inorganic phosphate.

The 5,10-methenyl and 10-formyl derivatives of tetrahydrofolate are interconvertible via a reversible reaction catalyzed by cyclohydrolase (*13*); nonenzymic catalysis of this reaction can also proceed at an appreciable rate:

$$\text{5,10-CH-FH}_4+\text{H}_2\text{O} \rightleftharpoons \text{10-CHO-FH}_4 \quad \text{(eq. 4)}$$

The synthetase from rabbit liver is inhibited by $\text{5-CH}_3\text{-FH}_4$, but the K_i is 5.5 μM, which compares unfavorably with the K_m of 0.5 μM for the substrate, 5-CHO-FH_4 (*14*). Cyclohydrolase from beef liver is inhibited by various folate compounds; 5-CHO-FH_4 has a K_i of 8.6 μM (*15*). The reported K_m values are 40 μM for 5,10-CH-FH_4 and 15 μM for 10-CHO-FH_4 (*15*).

The role of 5-CHO-FH_4 in eukaryotic cells is still obscure. Is it a stable storage form of the vitamin? Although this derivative is one of the major constituents of the intracellular folate pool (*16,17*), its route of synthesis is not yet clear. Since the ATP-dependent reaction (eq. 3) appears to be irreversible, formylation of tetrahydrofolate by *N*-formylglutamate would seem to be the only alternative route (*18*):

$$\text{FH}_4+N\text{-formylglutamate} \rightleftharpoons \text{5-CHO-FH}_4+\text{glutamate} \quad \text{(eq. 5)}$$

No physiological source of *N*-formylglutamate, however, has been identified.

5-CHO-FH_4 has been used extensively as a "rescue agent" in high-dose MTX therapy. It replenishes the reduced folate pool after dihydrofolate reductase has been blocked by MTX (fig. 5). However, the first step in the utilization of this substrate is not yet clear. It could be reaction 3 or reaction 5 in reverse, or a combination of both reactions. Activity of the enzyme catalyzing reaction 3 can be monitored spectrophotometrically (fig. 6), and a similar assay could be developed for reaction 5. Data on the levels of these enzymes in various tumors and normal tissues would be helpful in predicting clinical situations that are likely to respond favorably to high-dose MTX therapy with folinate rescue.

Purine Synthesis

10-CHO-FH_4 is the C_1 donor for both the C-2 and C-8 positions of inosine monophosphate. Glycinamide ribonu-

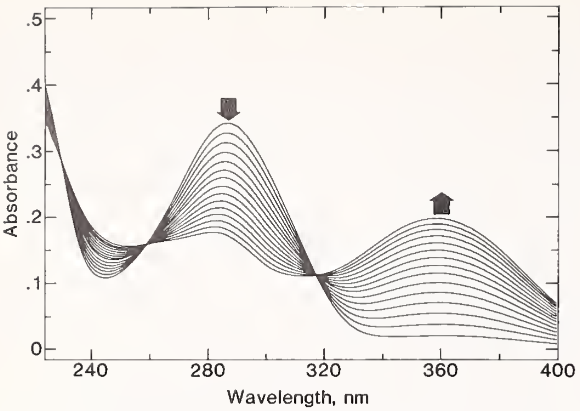


FIGURE 6.—Spectrophotometric demonstration of the enzymic conversion of 5-CHO-FH_4 to 5,10-CH-FH_4. The 1.0-ml reaction mixture [50 m*M* Mes buffer, 10 m*M* $Mg(acetate)_2$, 1 m*M* dithiothreitol, 20 μM ATP, 20 μM 5-CHO-FH_4, 0.1 m*M* EDTA, 0.9 milliunits 5,10-CH-FH_4 synthetase, pH 6.0] at 23°C was contained in a cuvet of 1-cm light path. The matched reference cuvet lacked enzyme and contained one-half (10 μM) the 5-CHO-FH_4 concentration to offset absorbance due to inactive diastereoisomer. Repetitive scans (60-sec period, 5-sec integration) were recorded automatically. Reprinted with permission from J Biol Chem 259:2728–2733, 1984.

cleotide (GAR) transformylase and 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) transformylase, which catalyze these reactions, have been purified extensively, and recently the mechanisms have been elucidated in the laboratories of Benkovic (*20*) and Stubbe (*21*):

10-CHO-FH_4 + GAR → tetrahydrofolate
+ formylglycinamide ribonucleotide (eq. 6)

10-CHO-FH_4 + AICAR → tetrahydrofolate
+ 5-formamido-4-imidazolecarboxamide
ribonucleotide (eq. 7)

The importance of purine nucleotides as constituents of DNA and RNA (as well as various coenzymes) makes these enzymes attractive targets for inhibition. However, most of the enzymes responsible for individual steps in the purine biosynthesis pathway, including the two transformylases, occur in clusters on multifunctional proteins (*20*). This makes it difficult for inhibitors to intervene when the substrate is channeled from one active site to another with minimal dissociation from the protein surface. The GAR and AICAR transformylases, purified from chicken liver, have K_m values of 8.9 and 68 μM, respectively, for 10-CHO-FH_4 (*20*). The former enzyme is inhibited by the 8-deaza analog of the substrate ($K_i = 4\ \mu M$), while the latter is inhibited by the 5,8-dideaza analog ($K_i = 29\ \mu M$). 11-CHO-FH_4 inhibits GAR transformylase, but not AICAR transformylase, with a K_i of 4 μM (*20*). The pentaglutamate of dihydrofolate, which accumulates in MCF-7 human breast tumor cells treated with MTX, is a potent inhibitor ($K_i = 0.043\ \mu M$) of AICAR transformylase (*22*); this can be compared to the K_m for the pentaglutamate of 10-CHO-FH_4, which is 5.5 μM. Thus, the cytotoxicity of MTX in these cells is due to a combination of inhibition of thymidylate synthesis (antipyrimidine effect) and inhibition of purine synthesis (antipurine effect).

Serine Synthesis

Serine hydroxymethyltransferase (*23*), which catalyzes the reversible reaction shown below is a potential target for chemotherapeutic agents:

5,10-CH_2-FH_4 + glycine $\rightleftharpoons$ tetrahydrofolate + serine (eq. 8)

This assumption is based upon the probability that reaction 8 in reverse is one of the main sources of C_1 units for the folate network. The importance of serine hydroxymethyltransferase as a chemotherapeutic target is further emphasized by the observation that the level of this enzyme is elevated approximately 10-fold in human leukemic leukocytes (*24*). Yet despite these favorable aspects, only a few useful inhibitors have been developed. Tetrahydrohomofolate has a K_i value of approximately 60 μM for the enzyme from L1210 cells (*25*), which may be compared to the K_m of 45 μM for tetrahydrofolate [determined using the homogeneous rabbit liver enzyme (*26*)]. Analogs of the non-folate substrate, β-D-fluoroalanine and D-cycloserine, are also reported to inhibit the enzyme (*23*).

Thymidylate Synthesis

This process involves a cyclic sequence (fig. 4) catalyzed by thymidylate synthetase, dihydrofolate reductase and serine hydroxymethyltransferase. The latter enzyme has been considered in the previous section, and the present discussion will focus only briefly upon the other two enzymes, which have been treated extensively elsewhere (*27,28*).

Thymidylate Synthetase

This enzyme catalyzes the irreversible reaction shown below:

5,10-CH_2-FH_4 + dUMP → dihydrofolate + dTMP (eq. 9)

Since thymidylate (as its triphosphate derivative) is considered to be a limiting component in DNA synthesis, this enzyme has received extensive attention as a target for cancer chemotherapy. The *Lactobacillus casei* enzyme has been crystallized and sequenced (*29*), but the structure has not been determined. Fluorodeoxyuridylate, the activated form of FUra (or of fluorodeoxyuridine), is an analog of the substrate deoxyuridylate. Its real potency, however, results from the fortuitous fact that the mechanism of the enzyme reaction causes the drug and the substrate, 5,10-CH_2-FH_4, to bind covalently to the enzyme and form an inactive enzyme-substrate inhibitor complex (fig. 7). Possibly because fluorodeoxyuridylate is such an effective inhibitor of the enzyme, less attention has been directed toward folate antagonists. However, 10-propargyl-5,8-dideazafo-

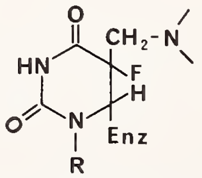


FIGURE 7.—Ternary complex of thymidylate synthetase (Enz) with 5,10-CH_3-FH_4 and fluorodeoxyuridylate. The methylene group (-CH_2-) is bridged between N-5 of tetrahydrofolate and C-5 of fluorodeoxyuridylate. From (*30*).

late is quite effective [$K_i = 0.005\ \mu M$ for the L1210 enzyme (*31*)]; this may be compared to the K_m for methylenetetrahydrofolate which has a value ranging from 5 to 50 μM for the enzyme from various sources (*27*). Methotrexate is only a weak inhibitor of thymidylate synthetases, compared to its ability to inhibit dihydrofolate reductases.

Dihydrofolate Reductase

This enzyme, which catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate is the most well studied of the folate-dependent systems:

$$\text{dihydrofolate} + \text{NADPH} \rightarrow \text{tetrahydrofolate} + \text{NADP} \quad \text{(eq. 10)}$$

It is the target for MTX and other 4-amino analogs of folate. Dihydrofolate reductase has been isolated from a variety of sources. In some instances the enzyme has been sequenced and crystallized, and the structure determined by X-ray diffraction analysis [reviewed in (*32*)]. In these latter cases, the fit of the drug in the active site of the enzyme can be visualized. The flexibility of the protein in solution, however, allows it to assume conformational forms (*33*) that differ from that existing in the crystal structure.

The success of MTX as a cancer chemotherapeutic agent has led to the synthesis of a large number of other folate analogs. Most of these that have shown good therapeutic potential in tumor screens are also good inhibitors of dihydrofolate reductase. Indeed, new MTX analogs should be tested first for enzyme inhibition, since those lacking this ability probably will not have any appreciable cytotoxicity. The synthesis of additional analogs, however, requires a careful consideration of the purpose for which they are to be used. MTX generally produces a satisfactory cell kill when used in the initial treatment of patients with responsive tumors. New analogs are probably not needed in these situations. But when cells have become resistant to the drug, new strategies and new compounds are required. These will vary depending upon whether resistance is due to: 1) decreased transport of the drug, 2) increased amount of the target enzyme, or 3) enzyme with decreased affinity for the drug. Defects in the transport system can be circumvented by using lipophilic analogs that enter cells by passive diffusion. Piritrexim (BW 301U) (fig. 8) is an example of this type of MTX analog. Resistance due to elevated amounts of dihydrofolate reductase is more difficult to overcome. MTX appears to induce gene amplification, which is responsible for overproduction of the enzyme. This drug, by prolonging the duration of cells in S phase, allows a portion of the genome (containing the dihydrofolate reductase gene) to be replicated repeatedly (*34*). Possibilities for treatment of this type of resistance, currently being considered in various laboratories, include: 1) Use of MTX linked to tumor-specific monoclonal antibodies to deliver large quantities of drug; 2) development of prodrug forms of MTX that would be activated and taken up selectively by tumor cells; and 3) synthesis of folate analogs that would be converted by the high levels of reductase to compounds inhibitory to other folate-dependent enzymes. Finally, resistance resulting from mutant forms of the enzyme, having reduced affinity for MTX, might be approached by determining the 3-dimensional structure of these enzymes and then designing new analogs that would better fit the altered binding site.

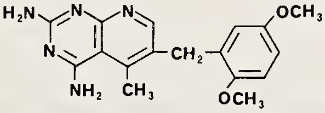


FIGURE 8.—Structure of piritrexim (BW 301U).

In addition to the classic 4-amino analogs of folate (i.e., MTX and its congeners) and truncated analogs of MTX, such as piritrexim (see above), analogs which bind covalently to the target enzyme represent a class of compounds that are still largely unexplored. Baker's antifols containing a terminal sulfonylfluoride (*35*) were the prototypes of this group (fig. 9). "Activated" folate compounds, produced by treatment of the precursors with carbodiimides (*36*) or *N*-hydroxysuccinimide (*37*), have been shown to bind covalently to membrane-associated, folate-carrier proteins, and they may also prove to be effective inhibitors of the enzyme. Since the stable complex formed by interaction of the diaquo form of cisplatin with tetrahydrofolate (*38*) reversibly inhibits the L1210 transport system and dihydrofolate reductase (unpublished observation of L. Pope and T. Duffy), the platinum tetrahydrofolate analog of the diaquo form of cisplatin (fig. 9) should be able to irreversibly inhibit these proteins.

Methionine Synthesis

This enzyme, which utilizes $5\text{-}CH_3\text{-}FH_4$ as the methyl donor for the conversion of homocysteine to methionine, contains a cobalamin coenzyme (*10*):

$$5\text{-}CH_3\text{-}FH_4 + \text{homocysteine} \rightarrow \text{tetrahydrofolate} + \text{methionine} \quad \text{(eq. 11)}$$

Since $5\text{-}CH_3\text{-}FH_4$ is generated by the irreversible reduction of $5,10\text{-}CH_2\text{-}FH_4$ (equation 2), it accumulates in B_{12} deficiency. Thus, when L1210 cells are made B_{12}-deficient by growth in the absence of serum [which furnishes B_{12} bound to transcobalamin II (TCII)], replication occurs if the cells are grown on folate or $5\text{-}CHO\text{-}FH_4$, but not on $5\text{-}CH_3\text{-}FH_4$ (*39*). Cell growth under the latter conditions resumes at the normal rate, however, when free or TCII-bound B_{12} is added (fig. 10).

In view of these observations, methionine synthetase must be considered as a prime target for exploitation in cancer chemotherapy. The enzyme is available for inhibition studies, having been purified to homogeneity from bacterial (*40*) and human (*41*) sources. Antagonists of folate, B_{12}, or *S*-adenosylmethionine (required for activation of the enzyme) would be potential inhibitors [reviewed in (*42*)].

FOLATE ANTAGONISTS: FUTURE PROSPECTS

The traditional approach to drug discovery has been to screen naturally occurring or synthetic products against a spectrum of tumors. Promising candidates serve as prototypes for the preparation of structural analogs that will show better cytotoxicity, selectivity, targeting, and pharmacologic characteristics. One can hardly fault this procedure, since it has led to the appearance of most of the compounds presently in clinical use. However, an alternate approach, "enzyme-targeted chemotherapy" (*43*), is gaining in popularity. This begins with the identification of enzymes that play an important role in cell replication.

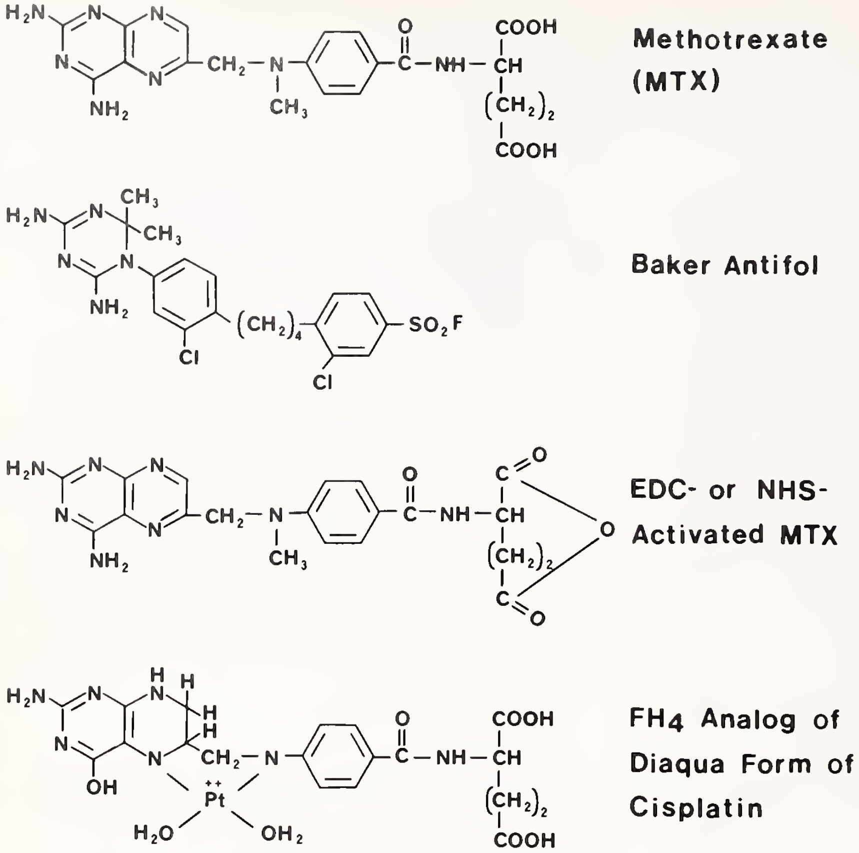

Methotrexate (MTX)
Baker Antifol
EDC- or NHS- Activated MTX
FH4 Analog of Diaqua Form of Cisplatin

FIGURE 9.—Candidate compounds for irreversible inhibition of dihydrofolate reductase. EDC = 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide; NHS = *N*-hydroxysuccinamide; FH_4 = tetrahydrofolate. For comparison, MTX is also shown.

These are generally rate-limiting in metabolic sequences and increased in activity in malignant cells. The enzymes are then purified to homogeneity, sequenced, crystallized, and subjected to X-ray diffraction analysis. Computer graphic modeling allows the binding sites to be visualized and analogs to be designed that will fit as well, or better, than the natural substrates.

Few compounds today are beyond the skill of the synthetic chemist. Attention must be paid, however, to the problem of introducing these compounds into cells. If they are polar, internalization usually requires transport by a system whose physiological substrate has similar structural characteristics. Alternatively, the analogs may be modified to make them less polar, thereby allowing entry into cells via diffusion. Finally, the compounds should be soluble, refractory to metabolism and/or excretion, able to locate tumor cells in sanctuaries, and relatively non-toxic toward normal cells.

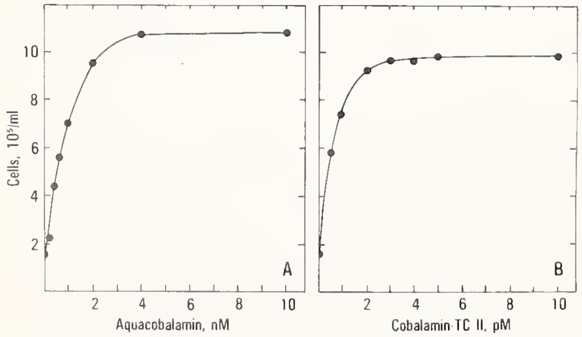


FIGURE 10.—Concentration dependence upon cobalamin (free and TC II-bound) for cobalamin-deficient L1210 cells growing on 5-CH_3-FH_4. 5-CH_3-FH_4 was present at 10 *µM*, and free aquacobalamin (panel A) or TC II-bound cobalamin (panel B) was present at indicated concentrations. Reprinted with permission from J Biol Chem 256:10329–10334, 1981.

Folate metabolism is one of the most promising areas for cancer chemotherapy. Folate-dependent enzymes play key roles in cell replication, and are present in relatively small amounts in cells. The extensive use of MTX and FUra in the treatment of human cancer clearly demonstrates how interference with two of these enzymes, dihydrofolate reductase and thymidylate synthetase, can produce impressive clinical results. Several of the other folate-dependent enzymes have great potential. The most promising candidates appear to be the two transformylases involved in purine synthesis, serine hydroxymethyltransferase, methylenetetrahydrofolate dehydrogenase, and methionine synthetase. These enzymes are available in highly purified form and ready for exploitation. Development of new agents, targeted against these enzymes, is a goal that is both desirable and attainable.

REFERENCES

(*1*) Orwell G: Animal Farm. New York: Harcourt Brace Jovanovich, 1946, p 123.

(*2*) Huennekens FM, Vitols KS, Henderson GB: Transport of folate compounds in bacterial and mammalian cells. *In* Advances in Enzymology (Meister A, ed). New York: John Wiley & Sons, 1978, pp 313–346.

(*3*) Goldman ID: Effects of methotrexate on cellular metabolism: Some critical elements in the drug-cell interaction. Cancer Treat Rep 61:549–558, 1977.

(*4*) Dembo M, Sirotnak FM: Membrane transport of folate compounds in mammalian cells. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WB, et al, eds), vol 1. New York: Academic Press, 1984, pp 173–217.

(*5*) Henderson GB: Transport of folate compounds into cells. *In* Nutritional, Pharmacologic and Physiologic Aspects of Folates and Pterins (Blakley RL, Whitehead MV, eds). New York: John Wiley & Sons, 1986, pp 207–250.

(*6*) Huennekens FM, Osborn MJ, Whiteley HR: Folic acid coenzymes. Science 128:120–128, 1958.

(*7*) Duch DS, Bowers SW, Nichol CA: Analysis of folate cofactor levels in tissues using high-performance liquid chromatography. Anal Biochem 130:385–392, 1983.

(*8*) Jackson RC, Harrap KR: Studies with a mathematical model of folate metabolism. Arch Biochem Biophys 158:827–841, 1973.

(*9*) Mackenzie RE: Biogenesis and interconversion of substituted tetrahydrofolates. *In* Folates and Pterins (Blakley RL, Benkovic SJ, eds). New York: John Wiley & Sons, 1984, pp 255–306.

(*10*) Matthews RG: Methionine biosynthesis. *In* Folates and Pterins (Blakley RL, Benkovic SJ, eds). New York: John Wiley & Sons, 1984, pp 497–553.

(*11*) Kutzbach C, Stokstad RL: Mammalian methylenetetrahydrofolate reductase. Partial purification, properties and inhibition by *S*-adenosylmethionine. Biochim Biophys Acta 250:459–477, 1971.

(*12*) Huennekens FM, Henderson GB, Vitols KS, et al: Enzymatic activation of 5-formyltetrahydrofolate via conversion to 5,10-methenyltetrahydrofolate. *In* Advances in Enzyme Regulation (Weber G, ed). Oxford: Pergamon Press, 1984, pp 3–13.

(*13*) Tabor H, Wyngarden L: The enzymatic formation of formiminotetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, and 10-formyltetrahydrofolic acid in the metabolism of formiminoglutamic acid. J Biol Chem 234:1830–1846, 1959.

(*14*) Hopkins S, Schirch V: 5,10-Methenyltetrahydrofolate synthetase. Purification and properties of the enzyme from rabbit liver. J Biol Chem 259:5618–5622, 1984.

(*15*) Rowe PB, Lewis GP: Mammalian folate metabolism. Regulation of folate interconversion enzymes. Biochemistry 12:1962–1969, 1973.

(*16*) Moran RG, Werkheiser WC, Zakrzewski SF: Folate metabolism in mammalian cells in culture. J Biol Chem 251:3569–3575, 1976.

(*17*) Fujii K, Nagasaki T, Vitols KS, et al: Polyglutamylation as a factor in the trapping of 5-methyltetrahydrofolate by cobalamin-deficient cells. *In* Folyl and Antifolyl Polyglutamates (Goldman ID, Chabner BA, Bertino JR, eds). New York: Plenum Press, 1983, pp 375–397.

(*18*) Silverman M, Keresztesy JC, Koval GJ, et al: Citrovorum factor and the synthesis of formylglutamic acid. J Biol Chem 226:83–94, 1957.

(*19*) Grimshaw CE, Henderson GB, Soppe GG, et al: Purification and properties of 5,10-methenyltetrahydrofolate synthetase from *Lactobacillus casei*. J Biol Chem 259: 2728–2733, 1984.

(*20*) Benkovic SJ: The transformylase enzymes in de novo purine biosynthesis. Trends Biochem Sci 9:320–322. 1984.

(*21*) Daubner SC, Schrimsher JL, Schendel FJ, et al: A multifunctional protein possessing glycinamide ribonucleotide synthetase, glycinamide ribonucleotide transformylase, and aminoimidazole ribonucleotide synthetase activities in de novo purine biosynthesis. Biochemistry 24:7059–7062, 1985.

(*22*) Allegra CJ, Drake JC, Jolivet J, et al: Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. Proc Natl Acad Sci USA 82:4881–4885, 1985.

(*23*) Schirch L: Folates in serine and glycine metabolism. *In* Folates and Pterins (Blakley RL, Benkovic SJ, eds). New York: John Wiley & Sons, 1984, pp 400–431.

(*24*) Bertino JR, Silber R, Freeman M, et al: Studies on normal and leukemic leukocytes. IV. Tetrahydrofolate-dependent enzyme systems and dihydrofolic reductase. J Clin Invest 42:1899–1907, 1963.

(*25*) Scanlon KJ, Cashmore AR, Moroson BA, et al: Inhibition of serine metabolism by tetrahydrohomofolate in L1210 mouse leukemic cells. Mol Pharmacol 19:481–490, 1981.

(*26*) Schirch L, Peterson D: Purification and properties of mitochondrial serine hydroxymethyltransferase. J Biol Chem 255:7801–7806, 1980.

(*27*) Santi DV, Danenberg PV: Folates in pyrimidine nucleotide biosynthesis. *In* Folates and Pterins (Blakley RL, Benkovic SJ, eds). New York: John Wiley & Sons, 1984, pp 347–398.

(*28*) Blakley RL: Dihydrofolate reductase. *In* Folates and Pterins (Blakley RL, Benkovic SJ, eds), vol 1. New York: John Wiley & Sons, 1984, pp 191–253.

(*29*) Maley GF, Bellisario RL, Guarino DUY, et al: The primary structre of *Lactobacillus casei* thymidylate synthetase. III. The use of 2-(2-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine and limited tryptic peptides to establish the complete amino acid sequence of the enzyme. J Biol Chem 254:1301–1304, 1979.

(*30*) Danenberg PV: Thymidylate synthetase: A target enzyme in cancer chemotherapy. Biochim Biophys Acta 473:73–92, 1977.

(*31*) Jones TR, Calvert AH, Jackman AL, et al: A potent antitumor quinazoline inhibitor of thymidylate synthetase: Synthesis, biological properties and therapeutic results in mice. Eur J Cancer 17:11–19, 1981.

(*32*) Freisheim JH, Matthews DA: The comparative biochemistry of dihydrofolate reductase. *In* Folate Antagonists and Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WB, et al, eds). Orlando: Academic Press, 1984, pp 70–131.

(*33*) Duffy TH, Beckman SB, Sato JK, et al: Polymorphism of dihydrofolate reductase from a methoxtrexate resistant subline of L1210 cells. Adv Enzyme Regul 23:3–12, 1985.

(*34*) Mariani BD, Schimke RT: Gene amplification in a single cell cycle in Chinese hamster ovary cells. J Biol Chem 259:1901–1910, 1984.

(*35*) Baker BR, Vermuelen NMJ: Irreversible enzyme inhibitors. CLXXVII. Active-site-directed irreversible inhibitors of dihydrofolate reductase derived from 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(phenylalkylphenyl)-*s*-triazine. J Med Chem 13:1154–1160, 1970.

(*36*) Henderson GB, Zevely EM, Huennekens FM: Irreversible inactivation of the methotrexate transport system of L1210 cells by carbodiimide-activated substrates. J Biol Chem 255:4829–4833, 1980.

(*37*) Henderson GB, Zevely EM: Affinity labeling of the 5-methyltetrahydrofolate/methotrexate transport protein of L1210 cells by treatment with an *N*-hydroxysuccinimide ester of $[^3H]$methotrexate. J Biol Chem 259:4558–4562, 1984.

(*38*) Grundler G, Huennekens FM: Interaction of cis-diamminediaquo platinum with tetrahydrofolate. Proc Am Assoc Cancer Res 26:260, 1985.

(*39*) Fujii K, Nagasaki T, Huennekens FM: Vitamin B_{12}-dependent replication of L1210 mouse leukemia cells: A model system for cobalamin-folate interrelationships. J Biol Chem 256:10329–10334, 1981.

(*40*) Fujii K, Huennekens FM: Activation of methionine synthetase by a reduced triphosphopyridine nucleotide-dependent flavoprotein system. J Biol Chem 249:6745–6753, 1974.

(*41*) Utley CS, Marcell PD, Allen RH, et al: Isolation and characterization of methionine synthetase from human placenta. J Biol Chem 260:13656–13665, 1985.

(*42*) Huennekens FM, DiGirolamo PM, Fujii K, et al: B_{12}-dependent methionine synthetase as a potential target for cancer chemotherapy. *In* Advances in Enzyme Regulation (Weber G, ed). Oxford: Pergamon Press, 1976, pp 187–205.

(*43*) Weber G (ed): Advances in Enzyme Regulation. Oxford: Pergamon Press, 1986.

Unresolved Issues in the Biochemical Pharmacology of Antifolates

Robert C. Jackson[1,*]

ABSTRACT—Despite extensive knowledge of the molecular basis for anticancer selectivity of antifolates, particularly classical antifolates, several fundamental questions remain unanswered. It is still not known why antifolate-treated cells die, rather than remain in stasis. The role of uracil misincorporation into DNA in causing irreparable damage has not yet been completely clarified, nor to what extent the antipurine effect of methotrexate (MTX) may be a desirable effect that contributes to antitumor activity. The antipurine effect may cause progression delay, with paradoxical "self-antagonism"; possibly the antipurine effect of MTX is a cause of toxic side effects. Even less is known about the molecular pharmacology of nonclassical antifolates. If they are not dependent for cellular uptake upon a neoplastic transformation-linked carrier, and since they are not subject to polyglutamylation, the molecular basis for anticancer selectivity of nonclassical antifols is unclear. The mechanism by which trimetrexate and metoprine are transported into cells is not known; if it is by passive diffusion, it is odd that resistance is sometimes associated with impaired drug uptake. Other unanswered questions are the mechanism of cross-resistance of doxorubicin-resistant cells to trimetrexate, and why the cytotoxic effect of trimetrexate, at low concentrations, is reversed by thymidine in the absence of purines. Questions also remain concerning antifolate inhibitors of thymidylate synthase (TS), such as how 5,8-dideaza-10-propargylfolic acid (CB3717) enters cells, and whether TS inhibitors will have activity against slowly growing tumors. These and related questions are discussed in relation to the design of optimal antifolate chemotherapy.—NCI Monogr 5:9–15, 1987.

Fifteen years ago, a conference entitled "Folate Antagonists as Chemotherapeutic Agents" (organized by JR Bertino and FM Huennekens) was held at the New York Academy of Sciences (*1*). Thirteen years later, Sirotnak et al. edited a 2-volume monograph with almost the identical title (*2*). On reading the proceedings of the earlier meeting, one is impressed that many of the questions raised were answered, at least partially, by the time the latter work was published. In 1971 it was still possible to question seriously whether dihydrofolate reductase (DHFR) was in fact the primary intracellular site of action of MTX. By 1984 this could be regarded as established beyond reasonable dispute (*3*). Many questions about the structure-activity relationships of DHFR inhibitors, such as why the 2,4-diamino structure causes such tight binding inhibition, can now be answered in convincing detail thanks to the elegant X-ray crystallography studies of enzyme-inhibitor complexes [see Freisheim and Matthews (*4*) for review]. In addition, numerous studies have elucidated the effects of antifolates on cellular ribonucleotide and deoxyribonucleotide pools (*5*). The new technique of flow cytometry has provided a wealth of information on the cell cycle effects of antifolates (*6,7*). The technique of computer simulation of metabolic pathways, whose first application to the study of anticancer drugs was reported by Werkheiser (*8*) in the 1971 New York Academy of Sciences volume, has suggested a plausible explanation for the requirement for free intracellular MTX for effective cytotoxicity (*9,10*). Another paper in the Annals volume, by Baugh and Krumdieck (*11*), discussed early data on folate polyglutamates. Subsequent work on polyglutamylation of MTX has enormously increased our understanding of its antitumor selectivity, and in addition provided an explanation of the previously puzzling question of why an S-phase specific agent, with a short plasma half-life, should be so effective when given on intermittent schedules. The causes of acquired resistance of tumors to MTX, partially elucidated in 1971, are now understood in considerable detail for experimental tumors (*12*).

Complete answers to several other questions about the biochemical pharmacology of antifolates are not yet available, although partial answers are. An example in this category is the molecular basis for the antitumor selectivity of MTX. More complete knowledge of MTX selectivity will require additional studies of MTX transport and metabolism in a variety of normal tissues. Another example is MTX resistance. Acquired resistance to MTX is less well understood in human tumors than in experimental systems. Well-documented examples are available of drug resistance due to DHFR gene amplification, loss of membrane transport, altered DHFR inhibition kinetics, and decreased MTX polyglutamylation. Less clear are the relative frequencies of these resistance mechanisms, the degree of resistance encountered in clinical situations, and the extent to which MTX resistance of human tumors may be overcome by the technique of high dose MTX followed by leucovorin rescue. Intrinsic insensitivity to MTX has also been attributed to abnormal DHFR, poor drug transport, slow polyglutamylation, and cytokinetic factors, but again our understanding of why certain kinds of tumor types rarely respond to antifolates is rather fragmentary.

The progress in the biochemical pharmacology of antifolates in the past 15 years certainly provides some grounds for satisfaction. These developments have been described in detail in recent reviews, most notably in the monograph by Sirotnak et al. (*2*), and will not be discussed further here.

ABBREVIATIONS: DHFR = dihydrofolate reductase; dNTP = deoxyribonucleoside triphosphate; IAHQ = 5,8-dideazaisofolic acid; MTX = methotrexate; PALA = *N*-(phosphonacetyl)-L-aspartate; TS = thymidylate synthase.

[1] Chemotherapy Department, Warner-Lambert/Parke-Davis Pharmaceutical Research, Ann Arbor, MI.

[*] *Reprint requests to:* Robert C. Jackson, Ph.D., Warner-Lambert/Parke-Davis Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor, MI 48105.

Instead, I shall consider several questions concerning antifolates that remain largely unanswered.

WHY DO ANTIFOLATE-TREATED CELLS DIE?

Antimetabolites may cause cytotoxicity or cytostasis. However, to be an effective anticancer agent, an antimetabolite must kill tumor cells, not merely hold them in a growth-arrested state until the drug concentration drops below its inhibitory threshold. Antifolates are both cytostatic and cytocidal. The exact conditions leading to cell death need to be delineated so that the antitumor effect may be maximized. At present we cannot define exactly which antifolate-induced biochemical imbalances cause cell death, rather than growth arrest, nor identify the irreparable lesions involved. Cohen and Barner (*13*) described a seminal observation on the antibacterial mechanism of sulfanilamide against *Escherichia coli* B. Sulfanilamide induced a thymineless and purineless state, and was bacteriostatic; however, sulfanilamide plus a purine induced a specifically thymineless state which Cohen termed "unbalanced growth", and was bactericidal. Unbalanced growth is a state in which synthesis of RNA and protein can continue, allowing cellular enlargement, but DNA synthesis, and consequently cell division, are blocked. Cohen (*14*) speculated about exactly how unbalanced growth might cause cell death, but we know very little more about this now than we did 15 years ago. Several studies attempted to determine whether these conclusions about sulfanilamide effects on *E. coli* also applied to effects of MTX on mammalian cells, in particular whether purines increased the degree of cytotoxicity. Early results were equivocal: purines sometimes seemed to enhance MTX cytotoxicity and sometimes protected cells from MTX. Interpretation of these early studies was often complicated by the presence of unknown and variable amounts of purines present in the undialyzed serum used to supplement growth media. Much of this confusion was dispelled by a study of Taylor et al. (*15*) who showed, in 3 cell lines, that the effect of hypoxanthine (or adenosine, or deoxyadenosine) depended upon the concentration of MTX: when MTX was below 80 n*M,* purines normalized the drug-induced deoxyribonucleoside triphosphate (dNTP) pool imbalance, and tended to protect cells from the MTX. At MTX concentrations above 80 n*M,* purines exaggerated the dNTP pool perturbation, and potentiated MTX cytotoxicity. Thus, in mammalian cells, as in bacteria, antifolate-induced cytotoxicity is associated with unbalanced growth. For cell death to occur, it appears to be necessary that the unbalanced state, which corresponds to a state of S-phase arrest, persist for several hours, estimated to range from 8 (*16*) to 16 hours (*17*).

What is the nature of the irreversible lesion that occurs when cells are maintained for many hours in unbalanced S-phase arrest? Such cells appear to lose viability in colony assays before obvious lesions become apparent in the cell membrane or other subcellular organelles. In light of the fact that the most dramatic biochemical changes seen are in the dNTP pools, it seems most likely that the earliest irreversible damage is to DNA. However, changes are seen in pools of ATP and GTP in MTX-treated cells, so other forms of metabolic damage cannot be entirely ruled out as the final lethal effect of MTX.

One attractive explanation for MTX toxicity is that irreversible DNA damage is caused by uracil misincorporation. Treatment of cells with MTX causes accumulation of dUMP (*18*). Fridland (*19*) pointed out that a major source of dUMP was the deoxycytidylate deaminase reaction. This enzyme is subject to allosteric inhibition by dTTP. A mathematical modelling study (*20*) indicated that under normal conditions dCMP deaminase was inhibited by more than 96%. Thus conditions that deplete dTTP, such as MTX treatment, cause extensive activation of dCMP deaminase, with overproduction of dUMP, at the expense of deoxycytidine nucleotides. Verhoef and Fridland (*21*) suggested that under conditions where the dUMP pool was extensively elevated, the pool of dUTP might also be expected to increase, and since DNA polymerases do not distinguish between dUTP and dTTP, incorporation of uracil into DNA might occur. Normally this is prevented by a dUTPase that keeps the steady-state dUTP concentration extremely low, but it was suggested that under conditions of extreme deoxyuridylate overproduction this dUTPase might be overwhelmed. Evidence for accumulation of dUTP in MTX-treated cells was reported by Goulian et al. (*22*), and this group also presented evidence for uracil misincorporation into DNA in MTX-treated cells (*23*). Sedwick et al. (*24*) demonstrated that metoprine also caused uracil misincorporation.

Cells have evolved a mechanism for deletion of uracil bases from DNA. Cytosine bases in DNA may spontaneously deaminate to form uracil. Since uracil base-pairs with adenine instead of guanine, such deamination would cause a point mutation. The DNA-uracil glycosylase cleaves the glycosidic bond of the deoxyuridine in DNA, leaving an apyrimidinic site, which is normally excised and repaired. In an antifolate-treated cell, however, the situation will differ in 2 important respects. Firstly, the deleted uracil would be opposite an adenine (instead of a guanine) on the complementary strand, and thus the cell will attempt to replace it with a thymine, rather than a cytosine. Secondly, the cell will attempt this repair process under conditions where the dNTP pools are perturbed by the antifolate; in particular, dTTP is depleted, and dUTP much higher than normal. Thus there is a possibility that the cell may delete an offending uracil residue, only to replace it with another. Are the strand breaks that must occur at these excision sites sufficient to cause cell death? This certainly seems plausible, but at present there is no clear information relating antifolate cytotoxicity to dUTP accumulation, or to activity of DNA-uracil glycosylase or dUTPase. Moreover, although the quinazoline antifolate, CB3717, caused dUTP accumulation, its cytotoxicity was not potentiated by added deoxyuridine, nor antagonized by *N*-(phosphonacetyl)-L-aspartate (PALA) or pyrazofurin, compounds that block uridylate (and deoxyuridylate) accumulation (*25*). Thus, although it is attractive to speculate that antifolate cytotoxicity is caused by irreversible DNA damage to the S-phase arrested cells, this theory is still unproven.

ANTITHYMIDYLATE AND ANTIPURINE EFFECTS OF DHFR INHIBITORS

Early studies of MTX effects concentrated on its antithymidylate activity, and even used culture media containing added purines to prevent purine depletion. However, it is clear that protection from MTX cytotoxicity in most cell lines requires both thymidine and a purine, and that MTX causes depletion of dATP, dGTP, ATP and GTP to various extents, as well as dTTP depletion (reviewed in ref. *5*). Hryniuk (*26*) suggested that for some tumors the antipurine effect of MTX might be its primary antitumor mechanism. There is some evidence that most normal tissues (except liver) have little capacity for de novo purine biosynthesis, whereas tumors probably derive most of their purines by de novo biosynthesis (*27*). Thus, de novo purine biosynthesis inhibitors have inherent anticancer selectivity, and DHFR inhibitors may act functionally as antipurine agents.

However, there are various lines of evidence that the situation is much more complicated than this. Several questions arise: Does the antipurine effect of DHFR inhibitors contribute substantially to antitumor activity in most systems? If so, is the anticancer activity related to inhibition of DNA synthesis, or RNA synthesis, or both? Is the antipurine effect responsible for toxicity to normal tissues? Bearing in mind the studies of Cohen with sulfanilamide in bacteria (*14*), is a selective depletion of thymidylate more cytotoxic than depletion of both thymidylate and purines? Consider the following observations: CB3717, which is a TS inhibitor with no antipurine effect (even though it is also a weak inhibitor of DHFR), is clearly cytotoxic and has experimental and clinical antitumor activity. Thus antifolates do not require antipurine activity to be cytotoxic to tumor cells. In L1210 cells growing in inhibited steady state conditions, the degree of growth inhibition by MTX correlated with the depletion of dTTP (*28*). Based upon 2-parameter flow cytometry studies, in which cells were stained for both RNA and DNA, Taylor and Tattersall (*7*) concluded that the primary cytotoxic mechanism of MTX was inhibition of DNA synthesis resulting from deprivation of thymidylate or purine or both. However, the same authors (*15*) concluded that at very high MTX concentration (0.1 m*M*), MTX had an antipurine effect that blocked progression of cells across the G_1–S boundary; under these conditions addition of hypoxanthine potentiated cytotoxicity.

MTX probably gives a selective antithymidylate effect in bone marrow. Howell et al. (*29*) showed that bone marrow contains high local concentrations of hypoxanthine. The megaloblastic state of the marrow in MTX-treated subjects is certainly consistent with an unbalanced growth condition. In mice, the dose-limiting toxicity of MTX is gastrointestinal. Straw et al. (*30*) demonstrated that MTX induced a purineless state in mouse gut 1 hour after drug administration, but it took 24 hours to establish purine deficiency in bone marrow. The MTX-induced antipurine effect may thus contribute to the normal tissue toxicity of this agent, but in humans, where the dose-limiting toxicity is myelosuppression, this effect may be much less significant than in mice. The recent synthesis of 5,10-dideazatetrahydrofolate, an inhibitor of glycinamide ribotide transformylase (*31*) should make it possible to study many aspects of effects of antifolate purine antagonists on normal and malignant cells.

A related question concerns the secondary G_1–S progression delay caused by MTX at high doses. If this block is caused by purine depletion (which appears to be the case, since hypoxanthine relieves the block), how does the purine get depleted? Presumably, activity of the de novo purine biosynthetic pathway is limited by lack of tetrahydrofolate cofactors. This implies that a cell in late G1 phase of the cell cycle must contain sufficient activity of TS to deplete the tetrahydrofolate pool. It is not clear that experimental results support this; one study (*32*) found that TS was highly S-phase specific, and another (*33*) suggested that, although molecules of TS were present throughout the cell cycle, in intact cells the enzyme was only active in S-phase.

Finally, should we expect S-phase specific agents to be active against slowly growing tumors? Evidence indicates that they sometimes are. Probably this will depend upon the cytokinetic properties of particular tumors. If a tumor is slow-growing because only a relatively small fraction of cells are in cycle, DHFR inhibitors will have little activity, since they will not be cytotoxic to the viable noncycling cells. However, if a tumor has a slow growth rate because of a high cell-loss factor, then DHFR inhibitors could be active, since a high proportion of viable cells should pass through S-phase during the treatment period.

BIOCHEMICAL PHARMACOLOGY OF NONCLASSICAL DHFR INHIBITORS

The history of nonclassical folate antagonists (which in most cases are more lipophilic than MTX) goes back more than 30 years. However, the recent development of trimetrexate and piritrexim has stimulated a resurgence of interest in the chemistry and pharmacology of nonclassical antifolates. Several recent review articles summarize what is known about these compounds (*34–37*) but many questions remain unanswered.

Recent studies have shown that the antitumor selectivity of classical antifolates may be explained partly by greater rates of membrane transport of such drugs in tumor cells relative to normal tissues, and partly by more rapid and extensive polyglutamylation in tumors than in normal tissues (*38,39*). For nonclassical antifolates, which enter cells by a different mechanism, and which cannot be trapped within cells as polyglutamates, antitumor selectivity cannot be attributed to either of these mechanisms. To what is the antitumor selectivity of such agents as trimetrexate and metoprine due? The possibility that inhibition of de novo purine biosynthesis might contribute to the antitumor selectivity of MTX was discussed above. Although it was concluded that this did not appear to be a major factor in MTX selectivity, it could be more important for lipophilic antifolates. This possibility warrants further study. Depletion of tetrahydrofolate cofactors in the presence of antifolates requires TS activity, and TS tends to be higher in malignant cells than in normal cells. Thus the proliferation- and malignancy-linked nature of TS could be a major contributor to the anticancer selectivity of nonclassical antifolates. Although lipophilic antifolates are not transported into cells through the carrier system used by MTX, there is growing evidence that these compounds do not simply enter cells by passive diffusion. This evidence is discussed below. Since acquired resistance to lipophilic antifolates may be associated with differences in rates of cellular uptake, it is at least conceivable that rates of drug uptake may differ between normal cells and susceptible tumor cells. The amount of drug retention may also differ between cell types. Nonexchangeable trimetrexate accumulates in cells to levels far exceeding the DHFR content. Perhaps this bound drug may act as a cellular reservoir.

The mechanism by which lipophilic antifolates enter cells remains controversial (*40*). Greco and Hakala found that uptake of metoprine into KB and Hep-2 human carcinoma cells was not temperature-sensitive, and showed no evidence of saturability at concentrations up to 0.2 m*M* (*41*). Similarly, Duch et al. (*42*) reported that the rate of uptake of piritrexim was the same at 4°C as at 37°C. Kamen et al. (*43*) studied trimetrexate transport in L1210 cells and found no effect of sodium azide or *p*-chloromercuribenzenesulfonic acid. However, Fry et al. (*40,44*) studied trimetrexate transport in WI–L2 human lymphoblasts and found 50% suppression of transport and net uptake by 10 m*M* sodium azide, and concentration-dependent inhibition of uptake by *p*-chloromercuribenzenesulfonic acid. In contrast, azide stimulated trimetrexate efflux, and this effect was partially reversible by glucose. Trimetrexate uptake in WI–L2 cells was temperature-sensitive with a temperature coefficient (Q_{10}) (27–37°C) of 2.44 and was completely abolished at 4°C. Although complete saturation did not occur, the increase in uptake rate deviated from linearity at concentrations above 0.5 m*M*. Perhaps the most suggestive evidence for facilitated transport of trimetrexate is the isolation of a 60-fold resistant subline of WI–L2 cells with impaired trimetrexate uptake. These cells were fully cross-resistant to metoprine and piritrexim, but only 3-fold resistant to methotrexate-γ-*tert*-butyl monoester (*40,44,45*). However, they were fully sensitive to doxorubicin and vincristine, indicating that the mutation causing resistance to the lipophilic antifolates was not a form of the well-documented pleiotropic resistance phenomenon. Rates of transport of MTX were identical in the trimetrexate-sensitive and -resistant WI–L2 cell lines. The inhibition constant for binding of trimetrexate by DHFR was unaltered in the resistant cells.

Resistance to trimetrexate associated with impaired drug transport has also been reported in a line of Molt-3 human leukemia cells (*46*), though these cells contained other abnormalities. Mini et al. (*47*), examining the correlation between intrinsic sensitivity to trimetrexate and drug uptake, reported that CCRF-CEM T-leukemia cells were 10-fold less sensitive to trimetrexate than were HCT-8 colon carcinoma cells, and that CCRF-CEM cells accumulated 10-fold less drug over a 4-hour period than did HCT-8 cells. This result was reminiscent of the earlier observation of Skeel et al. (*48*), that Walker 256 cells, which are highly sensitive to triazinate, accumulated that lipophilic antifolate to 50 times the level found in L1210 cells, which are much less sensitive than triazinate. Another example of impaired uptake for a nonclassical antifolate was reported by Hill et al. (*49*) who described decreased uptake of metoprine in a line of resistant L5178Y cells.

In the face of this incomplete and confusing collection of results, it is not possible to conclude with certainty that lipophilic antifolates are actively transported. If they are, there is no evidence as to what the physiological substrate for the carrier might be. It is, however, clear that there are many results that are not compatible with these compounds entering and leaving cells by simple passive diffusion. The cross-resistance data suggest that, if they are indeed transported, trimetrexate, metoprine, and piritrexim may utilize a common mechanism, but that MTX esters are taken into cells by a process different both from the mechanism that transports MTX itself and from the one that transports the nonclassical antifolates.

A different kind of resistance to lipophilic antifolates is that associated with pleiotropic drug resistance. Ramu et al. (*50*) reported that P388 cells resistant to doxorubicin were 5- and 34-fold cross-resistant, respectively, to the mono- and dimethyl esters of MTX. In our own laboratory (Fry DW, Klohs WD, unpublished observations), we have found that a pleiotropically resistant subline of P388 leukemia was cross-resistant to trimetrexate and to piritrexim and that resistance could be partially reversed by verapamil. Transport studies showed that net uptake of trimetrexate was depressed by 75% in the resistant cells, and that intracellular steady state levels could be restored by the presence of verapamil. The mechanism of pleiotropic resistance to lipophilic antifolates remains obscure. Although an active efflux carrier mechanism has been suggested, it is difficult to conceive a transport mechanism with binding specificity so broad as to embrace anthracyclines, vinca alkaloids, synthetic intercalating agents, and antifolates.

Study of the cellular pharmacokinetics of trimetrexate and other lipophilic antifolates reveals another striking difference between these compounds and the classical antifolates. The latter compounds accumulate in cells to levels somewhat above the concentration of DHFR in the cell. For example, in the case of MTX, treated cells contain a bound-drug fraction (roughly equal to the DHFR concentration), a small amount of free MTX (in a steady state with respect to the extracellular drug concentration), and variable amounts of MTX polyglutamates. In contrast, cells treated with trimetrexate often accumulate drug to a concentration greatly exceeding the cellular DHFR content, and much higher than the extracellular drug concentration. Much of this cellular trimetrexate is nonexchangeable, that is, it does not readily diffuse out when cells are transferred to drug-free medium. This raises 2 questions: To what is this nonexchangeable, non-DHFR-associated drug bound, and what is its functional significance? These questions are currently unanswered. The drug could be bound to a membrane fraction or to a protein; at present we do not even know where it is in the cell. It is possible that this nonexchangeable drug is slowly released to provide a reservoir of inhibitor available for binding to DHFR after extracellular drug levels decline, thus prolonging the effective inhibition time, but this too is unknown. Since the total accumulation of lipophilic antifolates differs widely between cell types and appears to correlate with drug sensitivity (*47,48*), this nonexchangeable drug retention may be related to the anticancer selectivity of these agents. It is clearly necessary to learn more about the intracellular location of this drug fraction and the nature of the material to which it binds.

A final question about trimetrexate concerns the extent to which its antipurine effect is necessary for cytotoxic activity. McCormack et al. (*51*) reported that thymidine could prevent the growth inhibition of L1210 cells caused by 20 n*M* trimetrexate. On the other hand, 1 μM trimetrexate had a pronounced antipurine effect, as shown by nucleotide pool measurements in WI-L2 cells (*37*). Thymidine (25 μM) did not protect WI-L2 cells from 1 μM trimetrexate, but we obtained complete protection from the growth inhibitory effect of 100 n*M* trimetrexate by thymidine, in agreement with the previous result of McCormack et al. (*51*). Thus, the antipurine effect of trimetrexate is concentration-dependent. It is not clear why this should be the case, since the depletion of cellular tetrahydrofolate cofactors consequent upon DHFR inhibition would be expected to limit biosynthesis of thymidylate and purines to approximately equal extents. Possibly the dialyzed serum used to supplement the growth medium contained residual traces of purines, or possibly intracellular RNA turnover provided sufficient purine to enable DNA synthesis and cell division to continue for 4 days at low trimetrexate concentration.

BIOCHEMICAL PHARMACOLOGY OF ANTIFOLATE THYMIDYLATE SYNTHASE INHIBITORS

The availability of folate antagonists with a 4-oxo group has made it possible to study the biochemical pharmacology of compounds that bind to TS at the 5,10-methylenetetrahydrofolate site. One such compound, 10-propargyl-5,8-dideazafolic acid (CB3717, ICI 155,387) has shown significant clinical anticancer activity. A related compound, 5,8-dideazaisofolic acid (IAHQ) is a more potent inhibitor of DHFR than of TS, but its triglutamate (a possible cellular metabolite) is a better TS inhibitor (*52*). A question still unanswered is how these compounds enter cells. Apparently CB3717 does not utilize the MTX carrier, since several studies have shown that cell lines highly resistant to MTX by virtue of transport defects are not appreciably cross-resistant to CB3717 (*53–55*). In addition, cells with decreased transport of trimetrexate were not cross-resistant to CB3717 (*40,55*). Since CB3717 is a close structural analogue of folic acid, it is possible that these two compounds enter cells by a common route. However, the mechanism or mechanisms by which folic acid itself enters cells is far from clear. Dembo and Sirotnak (*56*) concluded that most folic acid transport may be via a high-capacity, low-affinity carrier that is distinct from the MTX carrier. A cell line in which MTX transport was less than 2% of the normal value (*57*) had unchanged uptake of folic acid; careful purification of the radiolabelled folic acid used in this study precluded the possibility that the measured uptake was actually due to a contaminant. This mutant L1210 subline with almost total deletion of MTX transport, but normal folic acid uptake, was fully sensitive to CB3717. Uptake of CB3717 is certainly very slow. One possibility is that it enters cells by a process of fluid-phase endocytosis, as described for PALA (*58*); another is that the low-affinity folic acid carrier is at least partially responsible.

Another important question concerning antifolate TS inhibitors concerns their antitumor spectrum. This needs to be defined for human tumors to determine the clinical value of such compounds, but also for murine tumors, because of their importance as model systems in drug development. CB3717 has demonstrated clinical activity, particularly against cancers of breast and ovary, but it has little activity against most murine systems. It has been suggested that this discrepancy is due to the much higher thymidine level in mouse blood than in human blood; since quite low concentrations of thymidine prevent or reverse the effects of TS inhibition, the mouse is not, in general, a good test animal for TS inhibitors. CB3717 had curative activity against a tetraploid subline of L1210 leukemia, but much weaker activity against the NCI line of L1210, which is near-diploid. Possibly this difference is due to the greater thymidylate requirement of polyploid tumors. Ideally, TS inhibitors should be tested in a species whose plasma thymidine concentration is close to the human level. At present, little information is available on plasma thymidine levels for rats, hamsters, or other possible test animals. Interestingly, IAHQ has activity against murine colon carcinoma (*38*). It is not known whether this activity is the result of inhibition of TS or of DHFR; in the former case the activity should be blocked by administration of thymidine, and in the latter case it may not be.

WHAT IS THE BASIS FOR THE SYNERGISM BETWEEN ALKYLATING AGENTS AND ANTIFOLATES?

A number of effective clinical protocols incorporate MTX in combination with an alkylating agent, generally cytoxan. These clinical studies have been reviewed by Tattersall (*59*). Examples include MTX plus cytoxan and vincristine in treatment of Burkitt's lymphoma, MTX plus cytoxan, vincristine and prednisone for non-Hodgkin's lymphoma, and MTX plus cytoxan and 5-fluorouracil (sometimes plus additional agents) in the adjuvant chemotherapy of breast cancer. In some cases, at least, there is evidence that the efficacy of cytoxan-containing regimens was enhanced by addition of MTX.

While therapeutic synergism may be difficult to establish conclusively in clinical chemotherapy, well-designed experimental chemotherapy studies with murine tumors have clearly shown synergism between alkylating agents and antifolates. Goldin (*60*) described synergistic activity of MTX plus cytoxan and MTX plus *bis*-(chloroethyl)nitrosourea against subcutaneous implants of L1210 leukemia, a rather refractory site for this tumor system. Recent work has shown strong synergism between cytoxan plus trimetrexate and cisplatin plus trimetrexate combinations against far-advanced P388 leukemia (Leopold et al., this symposium). Synergistic antitumor effects with alkylating agents have been described for other classes of antimetabolites as well as for antifolates. The explanation for these drug–drug interactions is far from clear. Goldin (*60*), discussing the alkylating agents–antifolate synergy, suggested that it might result from the alkylating agent killing of G_0 cells that had been immune from the effects of the S-phase-specific MTX. This explanation is a plausible one, but there is a lack of definitive cytokinetic studies of these combinations. Another possibility is that antimetabolites, by depleting cellular dNTP pools, inhibit repair of alkylating agent induced DNA damage. Again, there is very little experimental evidence bearing on this question. A third contributory factor may be lack of mutual cross-resistance between the two classes of drugs, e.g., cytoxan-resistant tumor cells are unlikely to be cross-resistant to MTX, and vice versa. Further studies on the mechanism of alkylating agent-antifolate interactions would be of great value in designing more effective combination regimens.

CONCLUSIONS

The last decade has greatly increased our understanding of the biochemical pharmacology of antifolates. As yet, this information has not been reflected in the introduction of more effective antifolates into established clinical practice.

However, the encouraging clinical trials on CB3717, 10-ethyl-10-deazaaminopterin, piritrexim, and trimetrexate provide grounds for optimism that one or more of these agents may find an established place in the treatment of human cancer. In comparison with the classical DHFR inhibitors, our understanding of the action of TS inhibitors and nonclassical DHFR inhibitors is still very fragmentary. In the meantime, even newer classes of antifolates, such as glycinamide ribotide transformylase inhibitors, are appearing on the scene. Forty years after the introduction of aminopterin, the study of folic acid antagonists remains full of scientific interest and therapeutic promise.

REFERENCES

(*1*) Bertino JR (ed): Folate antagonists as chemotherapeutic agents. Ann NY Acad Sci 186:1–519, 1971.

(*2*) Sirotnak FM, Burchall JJ, Ensminger WD, et al (eds): Folate Antagonists as Therapeutic Agents, vol II. New York: Academic Press, 1984.

(*3*) Jackson RC: Biological effects of folic acid antagonists with antineoplastic activity. Pharmacol Ther 25:61–82, 1984.

(*4*) Freisheim JH, Matthews DA: The comparative biochemistry of dihydrofolate reductase. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WD, et al, eds), vol I. New York: Academic Press, 1984, pp 69–131.

(*5*) Jackson RC, Grindey GB: The biochemical basis for methotrexate cytotoxicity. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WD, et al, eds), vol I. New York: Academic Press, 1984, pp 289–315.

(*6*) Hill BT: Lethal and kinetic effects of DDMP (2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine). Eur J Cancer 16:147–157, 1980.

(*7*) Taylor IW, Tattersall MHN: Methotrexate cytoxicity in cultured human leukemic cells studied by flow cytometry. Cancer Res 41:1549–1558, 1981.

(*8*) Werkheiser WC: Mathematical simulation in chemotherapy. Ann NY Acad Sci 186:343–358, 1971.

(*9*) Jackson RC, Harrap KR: Studies with a mathematical model of folate metabolism. Arch Biochem Biophys 158:827–841, 1973.

(*10*) White JC, Mikulecky DC: Application of network thermodynamics to the computer modeling of the pharmacology of anticancer agents: A network model for methotrexate as a comprehensive example. Pharmacol Ther 15: 251–291, 1982.

(*11*) Baugh CM, Krumdieck CL: Naturally occurring folates. Ann NY Acad Sci 186:7–28, 1971.

(*12*) Albrecht AM, Biedler JL: Acquired resistance of tumor cells to folate antagonists. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WD, et al, eds), vol I. New York: Academic Press, 1984, pp 317–353.

(*13*) Cohen SS, Barner MD: Studies on the induction of thymine deficiency and on the effects of thymidine and thymidine analogues in *Escherichia coli*. J Bacteriol 71:588–597, 1956.

(*14*) Cohen SS: On the nature of thymineless death. Ann NY Acad Sci 186:292–301, 1971.

(*15*) Taylor IW, Slowiaczek P, Francis PR, et al: Purine modulation of methotrexate cytotoxicity in mammalian cell lines. Cancer Res 42:5159–5164, 1982.

(*16*) Ernst P, Killmann SA: Perturbation of generation cycle of human leukemic myeloblasts in vivo by methotrexate. Blood 38:689–705, 1971.

(*17*) Rueckert RR, Mueller GC: Studies on unbalanced growth in tissue culture: Induction and consequences of thymidine deficiency. Cancer Res 20:1584–1591, 1960.

(*18*) Tattersall MHN, Jackson RC, Connors TA, et al: Combination chemotherapy: The interaction of methotrexate and 5-fluorouracil. Eur J Cancer 9:733–739, 1973.

(*19*) Fridland A: Effects of methotrexate on deoxyribonucleotide pools and DNA synthesis in human lymphocytic cells. Cancer Res 34:1883–1888, 1974.

(*20*) Jackson RC: A kinetic model of regulation of the deoxyribonucleoside triphosphate pool composition. Pharmacol Ther 24:279–301, 1984.

(*21*) Verhoef VL, Fridland A: Nascent DNA molecules in thymidine deprived human lymphoblasts. *In* Abstracts, 11th International Congress of Biochemistry, 1979, p 38.

(*22*) Goulian M, Bleile B, Tseng BY: The effect of methotrexate on levels of dUTP in animal cells. J Biol Chem 255:10630–10637, 1980.

(*23*) Goulian M, Bleile B, Tseng BY: Methotrexate-induced misincorporation of uracil into DNA. Proc Natl Acad Sci USA 77:1956–1960, 1980.

(*24*) Sedwick WD, Kutler M, Brown OE: Antifolate induced misincorporation of deoxyuridine monophosphate into DNA: Inhibition of high molecular weight DNA synthesis in human lymphoblastoid cells. Proc Natl Acad Sci USA 78:917–921, 1981.

(*25*) Jackson RC, Jackman AL, Calvert AH: Biochemical effects of the quinazoline inhibitor of thymidylate synthetase, CB3717, on human lymphoblastoid cells. Biochem Pharmacol 33:3783–3790, 1983.

(*26*) Hryniuk WM: Purineless death as a link between growth rate and cytotoxicity by methotrexate. Cancer Res 32: 1506–1511, 1972.

(*27*) Jackson RC, Harkrader RJ: The contributions of de novo and salvage pathways of nucleotide biosynthesis in normal and malignant cells. *In* Nucleosides and Cancer Treatment (Tattersall MHN, Fox RM, eds). Sydney: Academic Press, 1981, pp 18–31.

(*28*) Kinahan JJ, Otten M, Grindey GB: Evaluation of ribonucleoside and deoxyribonucleoside triphosphate pools in cultured leukemia cells during exposure to methotrexate or methotrexate plus thymidine. Cancer Res 39:3531–3539, 1979.

(*29*) Howell SB, Mansfield SJ, Taetle R: Thymidine and hypoxanthine requirements of normal and malignant cells for protection against methotrexate cytotoxicity. Cancer Res 41:945–950, 1981.

(*30*) Straw JA, Talbot DC, Taylor GA, et al: Some observations on the reversibility of methotrexate toxicity in normal proliferating tissues. J Natl Cancer Inst 58:91–97, 1977.

(*31*) Moran RG, Taylor EC, Beardsley GP: 5,10-Dideaza-5,6,7,8-tetrahydrofolic acid, a potent antifolate inhibitory to de novo purine synthesis. Proc Am Assoc Cancer Res 26:231, 1985.

(*32*) Conrad AH: Thymidylate synthetase activity in cultured mammalian cells. J Biol Chem 246:1318–1323, 1971.

(*33*) Rode W, Scanlon KJ, Moroson BA, et al: Regulation of thymidylate synthetase in mouse leukemia cells. J Biol Chem 255:1305–1311, 1980.

(*34*) Hill BT, Price LA: DDMP (2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine. Cancer Treat Rev 7:95–112, 1980.

(*35*) Werbel LM: Design and synthesis of lipophilic antifols as anticancer agents. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WD, et al, eds) vol I. New York: Academic Press, 1984, pp 261–287.

(*36*) Hutchison DJ, Schmid FA: Experimental cancer chemotherapy with folate antagonists. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WD, et al, eds), vol II. New York: Academic Press, 1984, pp 1–22.

(*37*) Jackson RC, Fry DW, Boritzki TJ, et al: Biochemical pharmacology of the lipophilic antifolate, trimetrexate. Adv Enzyme Regul 22:187–206, 1984.

(*38*) Sirotnak FM, De Graw JI: Selective antitumor action of folate analogs. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WD, et al, eds) vol I. New York: Academic Press, 1984, pp 43–95.

(*39*) Fry DW, Anderson LA, Borst M, et al: Analysis of the role of membrane transport and polyglutamylation of methotrexate in the gut and the Ehrlich tumor in vivo as factors in drug sensitivity and selectivity. Cancer Res 43:1087–1092, 1983.

(*40*) Fry DW, Jackson RC: Membrane transport alterations as mechanism of resistance to anticancer agents. Cancer Surveys 5:47–79, 1986.

(*41*) Greco WR, Hakala MT: Cellular pharmacokinetics of lipophilic diaminopyrimidine antifolates. J Pharmacol Exp Ther 212:39–46, 1980.

(*42*) Duch DS, Edelstein MP, Bowers SW, et al: Biochemical chemotherapeutic studies on 2,4-diamino-6-(2,5-dimethoxybenzyl-5-methylpyrido[2,3-d]pyrimidine (BW301U), a novel lipid-soluble inhibitor of dihydrofolate reductase. Cancer Res 42:3987–3994, 1982.

(*43*) Kamen BA, Eibl B, Cashmore A, et al: Uptake and efficacy of trimetrexate, a non-classical antifolate in methotrexate-resistant leukemia cells in vitro. Biochem Pharmacol 33:1697–1699, 1984.

(*44*) Fry DW, Besserer JA, Boritzki TJ, et al: Impaired cellular uptake as a mechanism of resistance to lipophilic antifolates. Proc Am Assoc Cancer Res 26:340, 1985.

(*45*) Besserer JA, Fry DW, Boritzki TJ, et al: Studies on cellular resistance and membrane transport with the quinazoline antifolate, trimetrexate. Fed Proc 43:1779, 1984.

(*46*) Arkin H, Ohnuma T, Takemura Y, et al: Development and characterization of a trimetrexate-resistant human acute lymphoblastic leukemia cell line. Proc Am Assoc Cancer Res 26:25, 1985.

(*47*) Mini E, Sobrero A, Moroson BA, et al: Differential efficacy of trimetrexate and methotrexate on human colon carcinoma and human leukemia cells in vitro. Proc Am Assoc Cancer Res 26:230, 1985.

(*48*) Skeel RT, Sawicki WL, Cashmore AK, et al: The basis for disparate sensitivity of L1210 leukemia and Walker 256 carcinoma to a new triazine folate antagonist. Cancer Res 33:2972–2976, 1973.

(*49*) Hill BT, Price LA, Harrison SI, et al: Studies on the transport and distribution of diaminopyrimidines in L5178Y lymphoblasts in cell culture. Biochem Pharmacol 24:535–538, 1975.

(*50*) Ramu A, Fridkin M, Steinherz R: Cross resistance to esters of methotrexate in a doxorubicin-resistant subline of P388 murine leukemia. Cancer Chemother Pharmacol 15:31–34, 1985.

(*51*) McCormack JJ, Heusner JJ, Hacker MP, et al: Further pharmacological studies of 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl] quinazoline (TMQ). Proc Am Assoc Cancer Res 22:225, 1981.

(*52*) Fernandes DJ, Bertino JR, Hynes JB: Biochemical and antitumor effects of 5,8-dideazaisopteroylglutamate, a unique quinazoline inhibitor of thymidylate synthase. Cancer Res 43:1117–1123, 1983.

(*53*) Diddens H, Niethammer D, Jackson RC: Patterns of cross-resistance to the antifolate drugs trimetrexate, metoprine, homofolate and CB3717 in human lymphoma and osteosarcoma cells resistant to methotrexate. Cancer Res 43:5286–5292, 1983.

(*54*) Jackson RC, Besserer JA, Fry DW: Effects of extracellular concentration of folate or leucovorin on cellular sensitivity to trimetrexate, CB3717 or methotrexate. Fed Proc 43:950, 1984.

(*55*) Takemura Y, Ohnuma T, Arkin H, et al: Biological effects of CB3717 on human acute lymphocytic leukemia cell lines resistant to methotrexate or trimetrexate. Proc Am Assoc Cancer Res 26:230, 1985.

(*56*) Dembo M, Sirotnak FM: Membrane transport of folate compounds *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WD, et al, eds), vol I. New York: Academic Press, 1984, pp 173–217.

(*57*) Fry DW, Besserer JA, Boritzki TJ: Transport of the antitumor antibiotic CI-920 in L1210 leukemia cells by the reduced folate carrier system. Cancer Res 44:3366–3370, 1984.

(*58*) White JC, Hines LH: Role of endocytosis and lysosomal pH in uptake of *N*-(phosphonacetyl)-L-aspartate and its inhibition of pyrimidine synthesis. Cancer Res 44:507–513, 1984.

(*59*) Tattersall MHN: Clinical utility of methotrexate in neoplastic disease. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WD, et al, eds), vol II. New York, Academic Press, 1984, pp 165–189.

(*60*) Goldin A: Combination chemotherapy with folate antagonists. Ann NY Acad Sci 186:423–437, 1971.

Biochemical Factors in the Selectivity of Leucovorin Rescue: Selective Inhibition of Leucovorin Reactivation of Dihydrofolate Reductase and Leucovorin Utilization in Purine and Pyrimidine Biosynthesis by Methotrexate and Dihydrofolate Polyglutamates[1]

I. David Goldman[2,*] and Larry H. Matherly[2,3]

ABSTRACT—Recent studies have clarified the critical role that polyglutamylation plays in methotrexate (MTX) action. Polyglutamate derivatives of MTX bind to dihydrofolate reductase (DHFR) with affinities comparable to the monoglutamate, but their retention in cells results in a sustained block in tetrahydrofolate (FH$_4$) synthesis. One important element in the selectivity of MTX action is the preferential buildup and retention of these polyglutamyl forms in susceptible tumor cells as compared to host cells of the bone marrow or gastrointestinal mucosa. This selectivity in the accumulation of MTX polyglutamyl forms has now been further shown to play an important role in the selectivity of leucovorin rescue and may provide a unique new approach to nucleoside protection as well. This paper reviews the current understanding of the biochemical basis for leucovorin rescue and its selectivity. Important elements in leucovorin rescue are reactivation of DHFR with depression of cellular dihydrofolate (FH$_2$) and provision of folate substrate to circumvent the block in FH$_4$ synthesis. Selectivity of leucovorin rescue may be attributed to direct inhibition by MTX polyglutamyl forms, as well as FH$_2$ polyglutamates that accumulate in their presence, at the levels of thymidylate synthase and transformylation during purine nucleotide biosynthesis. The presence of cellular MTX polyglutamates impairs reactivation of endogenous DHFR activity by leucovorin metabolites, and the resultant maintenance of high cellular levels of cellular FH$_2$ and the polyglutamyl derivations of MTX impair the utilization of added FH$_4$ in susceptible tumor cells. This paper also develops the concept of "early" nucleoside protection in antifolate therapy. In this approach, nucleosides are administered simultaneously with a pulse of MTX to provide early host protection from the cytotoxic effects of modest doses of MTX. Cessation of protection occurs at a time when extracellular and intracellular monoglutamate has fallen to low levels, and the polyglutamyl forms of the drug are present in susceptible tumors but not in host tissues of the gut and bone marrow. Data are presented to demonstrate that increased doses of MTX can be administered in normal and tumor-bearing animal systems as well as in humans by this technique.—NCI Monogr 5:17-26, 1987.

Attempts to modulate the pharmacologic activity of MTX have derived from the expectation that the delivery of high doses of this drug would be beneficial if toxicity to the host could be selectively reduced by protecting agents. Early studies in murine tumor systems after the clinical introduction of aminopterin and MTX demonstrated the improved chemotherapeutic efficacy of high doses of these antifolates with leucovorin rescue as compared to lower doses of antifolate alone (*1,2*). This approach was subsequently introduced into the clinics and remains an important component of many chemotherapeutic regimens. This paper provides an overview of the historical rationale for, and the probable mechanisms of, leucovorin rescue and the basis for the selectivity of this approach. The concept of nucleoside protection in antifolate therapy is also considered. A recent novel approach is described which was designed to exploit differences in the biochemical pharmacology of MTX in tumor versus host tissues and provides one clear basis for the selectivity of nucleoside protection.

RATIONALE FOR HIGH-DOSE MTX CLINICAL REGIMENS WITH LEUCOVORIN RESCUE

An attractive approach in cancer therapeutics applied with many different agents, in particular the antimetabolites, is the escalation of drug dose in an attempt to circumvent natural or acquired drug resistance. This was initially possible with MTX because of the availability of leucovorin rescue to obviate toxicity to host tissues. For most drugs, however, specific protecting or rescue agents are not available. The opportunity to apply high-dose MTX came early because of the realization by Goldin and his co-workers that the administration of leucovorin could circumvent host toxicity and enhance the therapeutic efficacy of this agent in experimental systems (*1,2*). This discovery was fortuitous and its clinical application empirical since it came decades before there was a basic understanding of the biochemical pharmacology of the rescue phenomenon.

There are a variety of elements which make it attractive to employ MTX in high doses. One of the earliest recog-

---

ABBREVIATIONS: AICAR = aminoimidazolecarboxamide ribonucleotide; 5,10-CH$^+$-FH$_4$ = 5,10-methenyltetrahydrofolate; 5,10-CH$_2$-FH$_4$ = 5,10-methylenetetrahydrofolate; 5-CH$_3$-FH$_4$ = 5-methyltetrahydrofolate; 5-CHO-FH$_4$ = 5-formyltetrahydrofolate; DHFR = dihydrofolate reductase; FH$_2$ = dihydrofolate: FH$_4$ = tetrahydrofolate; GAR = glycinamide ribonucleotide; K$_i$ = Michaelis-Menten inhibition constant; MTX = methotrexate; TMQ = trimetrexate (2,4-diamino-5-methyl-6-[(3,4,5-trimethoxy-anilino)-methyl]quinazoline).

---

[1] Supported by research grant CA-39807 and training grant CA-09340 (*L. H. Matherly*) from the National Cancer Institute, National Institutes of Health.

[2] Departments of Medicine and Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA.

[3] Recipient of a Junior Faculty Research Award from the American Cancer Society.

* *Reprint requests to:* I. David Goldman, M.D., Division of Hematology/Oncology, Medical College of Virginia, Box 230, Richmond, VA 23298.

nized factors related to the need for high drug doses to achieve penetration of pharmacologic sanctuaries such as the testis or the central nervous system. Here permeability barriers are overcome by the passive diffusion of drug in the presence of high systemic drug concentrations. Likewise, in the presence of elevated MTX levels, drug should diffuse into malignant cells in the core of poorly vascularized solid tumors.

We now recognize a number of important factors which account for a requirement for high concentrations of MTX to achieve a pharmacologic effect on the cellular level as well. First, even in drug-sensitive tumors, because of the excess of DHFR only a small percentage of the enzyme activity (approximately 5%) is sufficient to sustain FH_4 synthesis (*3,4*). When the enzyme is inhibited, FH_2 substrate accumulates and competes with MTX for this small fraction of enzyme. Consequently, complete suppression of cellular FH_4 synthesis requires drug levels in excess of the DHFR binding capacity, orders of magnitude higher than the Michaelis-Menten inhibition constant (K_i) for drug binding (*3,5–7*). From these considerations any change within cells that necessitates an increased level of free drug to suppress enzyme activity will be associated with reduced drug sensitivity. For instance, when the DHFR level within the cell is increased, the fraction of enzyme sufficient to maintain FH_4 synthesis is decreased and a greater degree of saturation of the enzyme by MTX is required to suppress enzyme activity. This results in a requirement for higher levels of free intracellular drug to achieve a pharmacologic effect. Similarly, high drug levels are required when resistance is associated with a DHFR variant with reduced affinity for MTX or when the rate of thymidylate synthesis is reduced with a resultant diminished rate of FH_2 generation.

All these factors can be circumvented by raising the extracellular drug concentration to increase the free antifolate level within the cell. High extracellular drug levels will also be required when resistance is associated with reduced affinity of the transport carrier for MTX, or when resistance is associated with the deletion of these carriers. For the latter, high extracellular drug levels allow increased MTX penetration of cells by passive diffusion or, possibly, low-affinity mediated process(es).

Finally, there are important factors which limit the steady-state levels of MTX achieved within cells. Influx of MTX is saturable but efflux is first order. This asymmetry is the consequence of the coupling of energy metabolism to the MTX transport system (*8*). Hence, the free intracellular MTX concentration is a saturable function of the extracellular MTX concentration. Therefore, as extracellular MTX increases, the steady-state free intracellular drug level achieved does not rise in proportion (*9*). Following saturation of the MTX transport carrier, free intracellular drug can increase only as a function of transport by passive diffusion or a second mediated transport process. Moreover, electrostatic factors depress free intracellular MTX based upon the negative transmembrane electrical-potential difference and the anionic nature of the MTX molecule (*8*). Finally, an energy-dependent process (i.e., an efflux pump) markedly depresses the free intracellular MTX level (*10,11*). These often overlooked properties of MTX transport explain why any change in the requirement for free intracellular drug, i.e., amplification of DHFR, reduced affinity of enzyme for drug, etc., requires a rise in extracellular drug concentration far out of proportion to the required increase in the intracellular drug level.

A BASIS FOR SELECTIVITY OF LEUCOVORIN RESCUE WHEN TUMOR CELLS ARE ISOLATED FROM THE SYSTEMIC VASCULATURE OR HAVE IMPAIRED MEMBRANE TRANSPORT OF MTX

MTX and aminopterin utilize a membrane carrier in mammalian cells which primarily serves to transport folate cofactors into cells (*8*). When this transport carrier is deleted or mutates resulting in a decreased affinity for its substrates, there is not only impaired transport of antifolates but reduced transport of FH_4 cofactors as well (*12,13*). Hence, when leucovorin is adminstered in very low doses following MTX, it presumably penetrates normal host cells of the bone marrow and intestinal epithelium rapidly but its rate of transport into transport-resistant tumor cells is markedly reduced. Herein lies one important basis for the selectivity of leucovorin rescue on the cellular level. Intrinsic to this approach is the attainment of an appropriately low plasma level of leucovorin during rescue sufficient to meet the requirements of normal cells but insufficient to overcome the permeability barrier that isolates resistant tumor cells. This element of selectivity will extend to any compartment with an appreciable permeability barrier to MTX and leucovorin such as the central nervous system, testis, or the core of a poorly vascularized solid tumor. Here, high concentrations of MTX achieve sufficient drug levels in the isolated compartment to achieve a pharmacologic effect, while the subsequent low concentrations of leucovorin do not result in sufficient penetration of the compartment to rescue tumor cells.

One interesting and potentially important corollary of this concept was a series of experiments in which lipid-soluble antifolates were administered simultaneously with low levels of leucovorin to animals bearing MTX-resistant tumors associated with impaired carrier-mediated transport of both MTX and reduced folates (*13,14*). Here the lipid-soluble antifolate circumvented the transport barrier by entering tumor cells by passive diffusion while penetrating normal tissues rapidly as well. However, the low concentrations of leucovorin, while overcoming the metabolic block induced by the antifolate in susceptible host tissues, did not penetrate resistant tumor cell membranes sufficiently to circumvent the antifolate effect. It is this impaired transport of required tetrahydrofolates into tumor cells resistant to MTX that accounts for the collateral sensitivity of these cells to lipid soluble antifolates.

Until very recently, the selectivity of leucovorin rescue was explained solely on the basis of compartmentalization of tumor cells or transport defects in resistant cells which exclude low levels of this reduced cofactor. Within the last few years, however, studies on the mechanism of leucovorin rescue and the selectivity of this phenomenon have focused attention on intracellular events that suggest important determinants of selectivity at this level as well. This is described in the next section.

DHFR REACTIVATION IN LEUCOVORIN RESCUE

The classical view of leucovorin rescue was that this agent circumvents the block in de novo FH_4 synthesis induced by antifolates by providing sufficient reduced

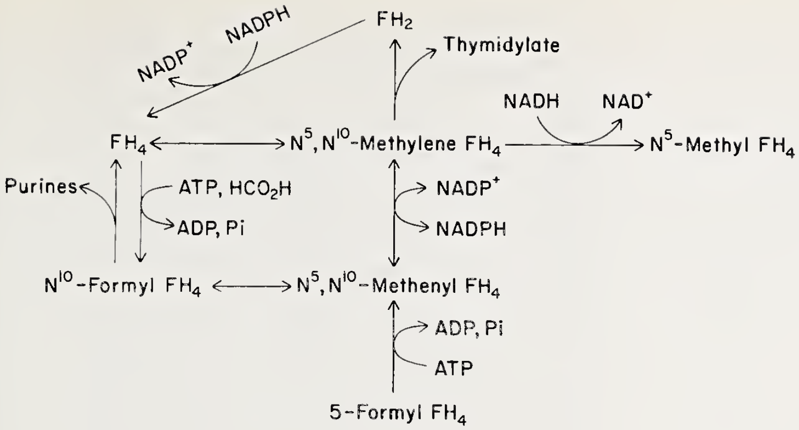


FIGURE 1.—Pathways of (6S)-5-CHO-FH$_4$ metabolism. Reprinted with permission from Cancer Res 46:588-593, 1986.

cofactor to cells to sustain folate-dependent reactions at a normal rate. As seen in figure 1, after transport into cells, 5-formyltetrahydrofolate (5-CHO-FH$_4$) is converted into 5,10-methenyltetrahydrofolate (5,10-CH$^+$-FH$_4$) which, in turn, is reduced to 5,10-methylenetetrahydrofolate (5,10-CH$_2$-FH$_4$). The latter is required for the biosynthesis of thymidylate and serine and is also reduced to 5-methyltetrahydrofolate (5-CH$_3$-FH$_4$) for methionine biosynthesis. In addition, 5,10-CH$^+$-FH$_4$ is readily converted through the action of the 5,10-CH$^+$-FH$_4$ cyclohydrolase, to 10-formyltetrahydrofolate (10-CHO-FH$_4$) which serves as the one-carbon moiety for purine nucleotide biosynthesis. Hence, leucovorin can, at least in theory, suffice as a source of reduced folates for cellular biosynthetic processes. However, White (*15*) hypothesized that during periods of maximum requirements for reduced folates, as in semiconservative DNA replication, transport of 5-CHO-FH$_4$ would be limiting to its utilization in the oxidation of 5,10-CH$_2$-FH$_4$ to FH$_2$ for thymidylate biosynthesis.

Recently, studies from this laboratory have provided new insights into the relationships between reduced folates and antifolates during leucovorin rescue (*16-19*). When cells are treated with MTX until DHFR is saturated, following which free intracellular MTX is eliminated, addition of 5-CHO-FH$_4$ results in the rapid net dissociation of antifolate from the enzyme (fig. 2, left panel). Moreover, bound MTX can be dissociated even when the formation of cellular FH$_2$ is inhibited in the presence of 5-fluoro-2′-deoxyuridine, indicating that this effect is not explained solely by competition between cellular FH$_2$ polyglutamates and MTX at the level of DHFR but may involve reduced cofactors as well. 5-CHO-FH$_4$ does not itself displace bound MTX (*15*); rather, this is related to cellular derivatives of this folate. Regardless of the cofactor form(s) involved in this response, the addition of 5-CHO-FH$_4$ to cells does not effect a net displacement of bound drug when the antifolate is present as intracellular polyglutamates (fig. 2, right panels).

MTX polyglutamates bind to DHFR with comparable affinities as monoglutamyl MTX (*20*). However, these polyglutamyl drug forms are selectively retained resulting in high intracellular drug levels even in the absence of extracellular MTX (*20-25*). In the presence of high intracellular levels of MTX polyglutamates, intracellular folates derived from leucovorin do not effectively compete with the drug for enzyme binding (*19*). Similar effects can be demonstrated in the presence of elevated levels of monoglutamyl MTX (*19*) or lipid-soluble antifolates such as trimetrexate (TMQ) (*18*) or 2,4-diamino-5-(3′,4′-dichlorophenyl)methylpyrimidine (*17*). Hence, a critical determinant of the extent of net drug displacement from DHFR in the presence of leucovorin appears to be the level of free antifolate in the vicinity of the target enzyme at the time rescue is initiated.

Of particular interest is the finding that 5-CHO-FH$_4$ supports only low levels of cell growth under conditions in which antifolate displacement from DHFR is not possible [i.e., in the presence of MTX polyglutamates or high concentrations of lipid-soluble antifolates; (*16-18*)]. Figure 3 presents data which suggest that reversal of antifolate growth inhibition by leucovorin is associated with reactivation of cellular DHFR. Growth inhibition despite the presence of leucovorin is correlated with the net accumulation of tritiated FH$_2$ formed from radiolabeled (6S)-5-CHO-FH$_4$. In the presence of MTX polyglutamates, (formed dur-

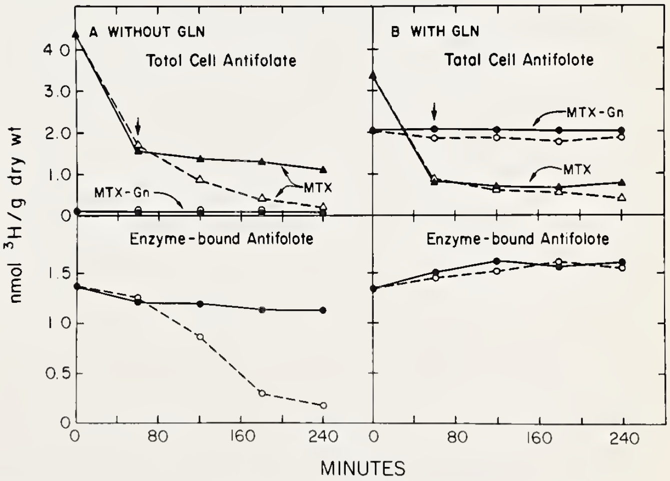


FIGURE 2.—Effect of leucovorin on DHFR-bound drug in the absence (left panels) or presence (right panels) of MTX polyglutamates within cells. Ehrlich ascites tumor cells were exposed to 5 μM tritiated MTX for 15 min (left), or 5 μM tritiated MTX and 5 mM glutamine (GLN) for 3 hr (right). The cells were then washed and resuspended into fresh drug-free medium. After 60 min (indicated by arrow), leucovorin (100 μM) was added to portions of the cell suspensions (represented by *stippled lines*) and the total intracellular MTX and its polyglutamate derivatives (MTX-Gn) were monitored (*upper panels*) along with the antifolate bound to DHFR (*lower panel*). Reprinted with permission from Cancer Res 43:2694-2699, 1983.

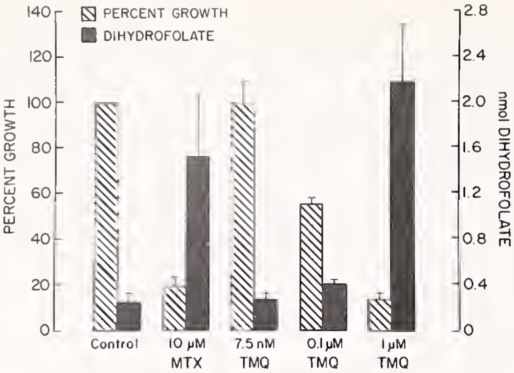


FIGURE 3.—The relationship between reduction of antifolate effects on cell growth by leucovorin and the cellular DHFR activity. Cells were incubated with tritiated (6S)5-CHO-FH$_4$ (5 μM) for 3 hr and the cellular folates extracted and quantitated by high-pressure liquid chromatography. Radiolabelled FH$_2$ is indicated on the right ordinate. The data reflect the mean (±SD) from duplicate experiments. The extent of growth inhibition was determined in the presence of 10 μM calcium leucovorin after a 3-hr pre-exposure to MTX (10 μM), following which MTX was removed, or in the continuous presence of TMQ (1 μM). Growth was monitored after 48 hr. The results are expressed as the mean (±SD) from 4 incubations in 2 separate experiments. Reprinted with permission from Cancer Res 46:588–593, 1986.

ing a pre-exposure to 10 μM drug), or in the continuous presence of 1 μM TMQ, leucovorin supports only low levels of growth (15–25%). This correlates with the accumulation of high concentrations of FH$_2$, reflecting suppression of DHFR activity under these conditions. However, when leucovorin appreciably reverses the effects of lower equitoxic TMQ concentrations (7.5 nM or 0.1 μM TMQ), there is a proportional reduction in the level of radiolabeled FH$_2$ formed, consistent with reactivation of DHFR under these conditions.

The focus to this point has been on DHFR as the primary target for 4-aminoantifolates and the role of competitive interactions at this locus in rescue. Another dimension of the rescue phenomenon is suggested by recent studies which indicate that treatment with 4-aminoantifolates results in the inhibition of enzyme sites in addition to DHFR within cells. This is considered in the next section.

INHIBITORY EFFECTS OF 4-AMINOANTIFOLATES AT ENZYME SITES DISTINCT FROM DHFR

There has been considerable speculation about possible effects of MTX at sites other than DHFR. Early studies identified MTX inhibition at the level of thymidylate synthase (*26*). However, the reported binding constants were generally too high to be considered pharmacologically important. With the recognition that MTX and other 4-aminoantifolates are converted to polyglutamates in cells, and that these conjugated drug forms, in general, have greater affinities for folate-dependent enzymes, including thymidylate synthase (*27*), aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase (*28–30*) and glycinamide ribonucleotide (GAR) transformylase (*30*), it was important to reconsider the pharmacologic significance of these drug interactions. Moreover, evidence has been presented from a number of laboratories for tight binding by FH$_2$ polyglutamates to various enzymes including thymidylate synthase (*31*), AICAR transformylase (*28,29*), and 5,10-CH$_2$-FH$_4$ reductase (*32*). The affinities of these FH$_2$ derivatives for these enzymes are often of sufficient magnitude to warrant serious consideration of their role in mediating aspects of antifolate activity.

Recent studies that characterize the metabolism of exogenous, stereospecific radiolabeled 5-CHO-FH$_4$ have enhanced our understanding of the complexity of 4-aminoantifolate action and suggest the extent to which inhibitory effects at the level of enzymes mediating reduced cofactor utilization can influence drug activity. Incubation of cells previously depleted of endogenous folate (by growth in folate-free medium containing 200 μM glycine, 100 μM adenosine, and 10 μM thymidine), with an appropriately radiolabeled derivative of 5-CHO-FH$_4$ permits an assessment of reduced cofactor interconversion and one-carbon metabolism, uninfluenced by the presence of endogenous folates. In this fashion, antifolate effects at specific folate-dependent steps can be evaluated.

In the absence of antifolate, (6S)-5-CHO[3′,5′,7,9-^{3}H]FH$_4$ is rapidly metabolized to 10-CHO[^{3}H]FH$_4$. After 3 hours of incubation of L1210 cells with 5-CHO-FH$_4$ (5 μM), greater than 89% of the metabolites formed were identified both intracellularly and extracellularly as 10-CHO-FH$_4$ (fig. 4). Only small quantities of other folates, including 5-CH$_3$-FH$_4$, 5,10-CH$^+$-FH$_4$, FH$_4$, and FH$_2$ were identified. When L1210 cells are either preloaded with MTX polyglutamates, or continuously exposed to TMQ (1 μM), there is an appreciable rise in the FH$_2$ levels (10- to 20-fold). However, there is only a small decline in the total 10-CHO-FH$_4$

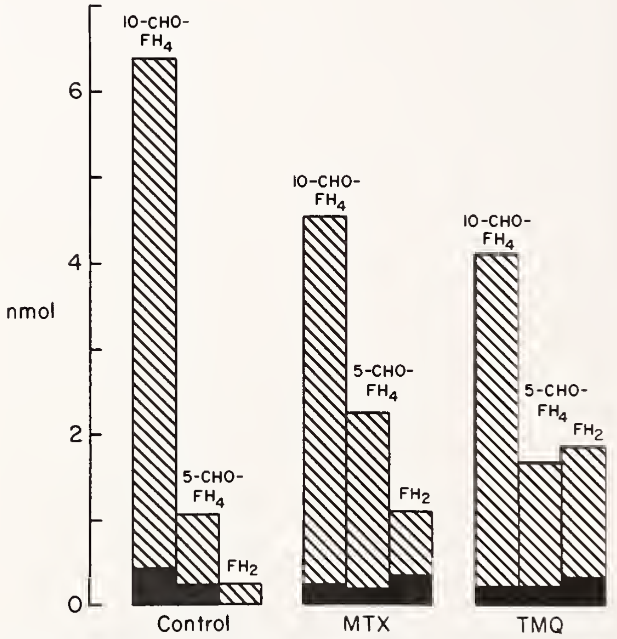


FIGURE 4.—Total intracellular and extracellular metabolites of (6S)-5-CHO [^{3}H] FH$_4$ in L1210 cells. Folate-depleted L1210 cells, either untreated or exposed to the antifolates, were incubated with 5 μM (6S)-5-CHO-[^{3}H]FH$_4$ for 3 hr followed by the determination of the major intracellular (*solid bars*) and extracellular (*striped bars*) folate derivatives. Only low levels of FH$_4$ and 5-CH$_3$-FH$_4$ were detected under these conditions. Reprinted with permission from Cancer Res 46: 588–593, 1986.

TABLE 1.—Carbon transfer from 5-[^{14}C]CHO-FH$_4$, [^{14}C]formate, or [^{14}C]serine into purine and thymine nucleotides

1-Carbon source[a]	Treatment	Thymine[b]	Purines[b]
5-[^{14}C]CHO-FH$_4$	None	7	134
5-[^{14}C]CHO-FH$_4$	TMQ(1 μM)	7	107
5-[^{14}C]CHO-FH$_4$	MTX(10 μM)	3	69
[^{14}C]Formate	None	94	861
[^{14}C]Formate	TMQ(1 μM)	26	264
[^{14}C]Formate	MTX(10 μM)	20	167
[3-^{14}C]Serine	None	1,394	9202
[3-^{14}C]Serine	TMQ(1 μM)	381	2129
[3-^{14}C]Serine	MTX(10 μM)	126	1076

[a] Folate depleted cells, treated as described in the text, were incubated with 5-[^{14}C]CHO-FH$_4$ (5 μM) for 90 min, or with [^{14}C]formate (100 μM), or [3-^{14}C]serine (285 μM) in the presence of (6s)5-CHO-FH$_4$ for 1 hr. Incorporation of the radioactive one carbon into nucleotides was determined by high-pressure liquid chromatography following acid hydrolysis of the total cell nucleotides to free bases.

[b] nmol/g dry weight.

accumulated from 5-CHO-FH$_4$ under these conditions (fig. 4).

Further studies have analyzed the effects of 4-aminoantifolates on the levels of incorporation of the radiolabeled one carbon from 5-[^{14}C]-CHO-FH$_4$ into cellular purine nucleotides and thymidylate in folate-depleted L1210 cells [(*33*); table 1]. Under these conditions any inhibitory effects on ^{14}C utilization are due to direct inhibition of folate requiring enzymes since the labeled one carbon is carried on the FH$_4$ molecule and there are negligible intracellular folates present. Incorporation of ^{14}C from 5-[^{14}C]CHO-FH$_4$ into thymidylate is essentially unperturbed in cells continuously exposed to TMQ (1 μM; table 1). However, this process is markedly suppressed in cells containing MTX polyglutamates generated during a preexposure to drug (10 μM; 3 hr). Likewise, the presence of cellular MTX polyglutamates is associated with inhibition of ^{14}C transfer from 5-[^{14}C]CHO-FH$_4$ into purine nucleotides. The continuous presence of TMQ (1 μM) results in a lesser inhibition of ^{14}C incorporation into newly synthesized purine nucleotides.

Hence, both MTX polyglutamates and TMQ appear to inhibit the unidirectional fluxes of one-carbon units from 5-[^{14}C]CHO-FH$_4$ into biosynthetic products in these folate-depleted cells. Since pretreatment with MTX or continuous exposure to 1 μM TMQ effects a comparable suppression of cellular DHFR under these conditions with a corresponding rise in cellular FH$_2$ (fig. 3), these data imply direct inhibition of thymidylate synthase by MTX polyglutamates. Polyglutamyl derivatives of FH$_2$ also inhibit thymidylate synthase in cell-free systems (*31,34*); however, the finding that TMQ had no effect on carbon transfer into thymidylate strongly suggests that this interaction is of little importance in mediating the effects of MTX on thymidylate biosynthesis. However, only limited buildup of FH$_2$ polyglutamates would be expected under the conditions of these experiments, possibly not sufficient to inhibit thymidylate synthase. In contrast to the effects on thymidylate biosynthesis, carbon transfer for purine biosynthesis is inhibited by both TMQ and MTX treatments. These findings are consistent with direct effects of these agents at the level of the GAR or AICAR transformylases and/or inhibition of these enzyme sites by FH$_2$ polyglutamates that accumulate in their presence.

A very different pattern of ^{14}C incorporation into biosynthetic products emerges when folate-depleted cells are incubated with unlabeled 5-CHO-FH$_4$ and folate-dependent biosyntheses are assessed by labelling the endogenous one-carbon pools with [^{14}C]formate or [^{14}C]serine. In both cases, utilization of the radiolabeled one-carbon moiety in biosynthetic reactions requires an initial association with unlabeled FH$_4$ that must be generated from the exogenous 5-CHO-FH$_4$ (fig. 1) to produce 10-[^{14}C]CHO-FH$_4$ and 5,10-[^{14}C]CH$_2$-FH$_4$, respectively. Under both these conditions, the presence of intracellular MTX polyglutamates or the continuous presence of 1 μM TMQ potently inhibits ^{14}C incorporation into purine nucleotides and thymidylate (table 1) suggesting a marked depletion of the FH$_4$ pool. This occurs even though the 10-CHO-FH$_4$ pool is only minimally perturbed (fig. 4). Similar suggestions of a depletion of FH$_4$ and 5,10-CH$_2$-FH$_4$ pools by MTX treatment can be found in the rapid inhibition by MTX of deoxyuridine incorporation into DNA in tumor cells containing normal levels of cellular folates (*5,7,33*). Alternatively, rapid buildup of FH$_2$ polyglutamates might feedback inhibit thymidylate synthase without depletion of 5,10-CH$_2$-FH$_4$ as suggested by Allegra et al. (*34*).

RAMIFICATIONS OF ANTIFOLATE-INDUCED INHIBITORY EFFECTS AT ENZYME SITES OTHER THAN DHFR

These studies of folate metabolic fluxes provide some important new insights into the mechanism of action of the 4-aminoantifolates. First, inhibition of cellular DHFR by TMQ or MTX polyglutamates is not accompanied by a quantitative conversion of reduced folates derived from 5-CHO-FH$_4$ to FH$_2$ during the biosynthesis of thymidylate. While it is not presently clear as to what extent this effect may be influenced by the level of ongoing thymidylate biosynthesis and, hence, the number of dividing S-phase cells in the heterogeneous cell population (*35*), it is of interest that the FH$_4$ pool is apparently depleted in drug-treated cells, even when considerable levels of 10-CHO-FH$_4$ have accumulated. This implies that the 10-CHO-FH$_4$ generated from 5-CHO-FH$_4$ is only minimally converted to FH$_4$ under these conditions. This lack of cofactor interconversion could arise from the unfavorable equilibrium of the 5,10-CH$^+$-FH$_4$ cyclohydrolase reaction which favors formation of 10-CHO-FH$_4$ (*36*) and the inhibition of one or both of the folate-dependent purine biosynthetic enzymes which generate FH$_4$, as described above. Studies from another laboratory have demonstrated preservation of endogenous tetrahydrofolate cofactor pools in the presence of MTX in cells equilibrated with tritiated folic acid (*37*).

However, the direct effects of MTX polyglutamates on folate-dependent enzymes distinct from DHFR might not necessarily augment antifolate activity. Rather, because of the slow rate of accumulation of appreciable levels of MTX polyglutamates, no significant direct inhibition of thymidylate synthase by these derivatives would arise until this activity is already reduced due to FH$_4$ depletion following DHFR inhibition by the monoglutamyl form of MTX. However, the elevated cellular FH$_2$ polyglutamates under these conditions would inhibit the conversion of 10-CHO-

FH_4 to FH_4, limiting the generation of 5,10-CH_2-FH_4 for thymidylate biosynthesis and could directly inhibit thymidylate synthase (*31,34*). While MTX polyglutamates also directly inhibit de novo purine biosynthesis, it is not clear to what extent this interaction enhances that attributable to the intracellular FH_2 polyglutamates which accumulate under these conditions. These effects at the level of the purines may augment antifolate effectiveness; however, diminished purine synthesis might also antagonize drug pharmacologic activity by "balancing" the levels of de novo synthesized purine nucleotides to the depressed thymidylate concentrations under these conditions (*38*). What is clear, as described in the next section, is that the buildup of MTX polyglutamates within cells plays an important role in limiting reduced cofactor utilization during leucovorin rescue and, therefore, may represent an important factor in the selectivity of the rescue phenomenon.

POLYGLUTAMYLATION OF ANTIFOLATES AS A BIOCHEMICAL BASIS FOR THE SELECTIVITY OF LEUCOVORIN RESCUE

Earlier studies demonstrated the importance of the polyglutamylation of 4-aminoantifolates in the selectivity of antifolate action. Hence, bone marrow cells, in general, and colony-forming units in particular, along with intestinal epithelial cells do not form appreciable levels of MTX polyglutamate derivates (*39–42*). This is in contrast to the accumulation of these metabolites in tumors highly susceptible to MTX (*39,40,42*). This differential metabolism provides an important selective advantage for host cells since after administration of drug, the block in FH_4 synthesis is transient in these host tissues and parallels the rise and decline of intracellular and plasma monoglutamyl MTX (*40*). However, the block in FH_4 synthesis in drug-sensitive tumor cells, while initiated by the monoglutamate, is sustained for long intervals after the underivatized MTX levels within and outside of cells have declined. This is due to the formation and persistence of active polyglutamyl derivatives which maintain suppression of DHFR activity.

The polyglutamylation of MTX may play a key role in the selectivity of leucovorin rescue as well. During rescue, bone marrow and gastrointestinal mucosa are exposed to leucovorin at a time when free intracellular MTX monoglutamate is declining. Under these conditions a number of interactions between MTX and leucovorin would occur. First, leucovorin would compete with MTX at the level of the transport carrier, further reducing the intracellular concentration of MTX (*8*). Second, once present intracellularly, leucovorin is rapidly metabolized, generating cofactor forms which compete with MTX for binding to DHFR. This results in the displacement of MTX from the target enzyme with restoration of enzyme activity. FH_2 returns to its usual low level, relieving the purine biosynthetic enzymes (and possibly thymidylate synthase as well) of any inhibition by this oxidized derivative. In this fashion, the synthesis and utilization of cellular reduced cofactors are returned to normal.

In cells which have accumulated MTX polyglutamates, however, leucovorin metabolites do not result in the net displacement of antifolate from DHFR. This results in a prolonged suppression of endogenous FH_4 synthesis and prolonged elevations of intracellular FH_2 polyglutamate levels. Under these conditions, the purine biosynthetic pathway is inhibited by both the polyglutamyl forms of FH_2 and MTX. Thymidylate synthase activity is also inhibited both directly by the conjugated MTX forms, the dihydrofolate polyglutamates that accumulate in its presence, and also through the depletion of 5,10-CH_2-FH_4 pools arising from the block at the level of purine biosynthesis. Hence, inhibitory effects at several levels appear to contribute to the inability of leucovorin to reverse antifolate pharmacologic activity in cells which have accumulated significant amounts of MTX polyglutamates.

NUCLEOSIDES AS SELECTIVE PROTECTING AGENTS

An alternative approach to the prevention of antifolate activity is the administration of the end-products of folate dependent reactions—purines, thymidine, and amino acids. In mice, a purine and thymidine are required to prevent or reverse the toxic effects of MTX (*43*). In man, however, thymidine alone reverses MTX toxicity (*44,45*). The clinical efficacy of high-dose MTX with thymidine protection/rescue has been explored in some detail. A variety of approaches have been evaluated including simultaneous infusions of MTX and thymidine, and MTX infusions (24 to 36 hr) followed by thymidine rescue (*44–46*). No clear efficacy could be demonstrated in these studies, but these regimens were not based upon any specific pharmacologic rationale for achieving antitumor selectivity.

Recently, studies from this laboratory have attempted to exploit the difference in polyglutamylation of MTX in drug-sensitive tumors versus susceptible host cells of the mouse in the development of a novel approach to nucleoside protection. Figure 5 demonstrates the difference in the accumulation and decline of MTX and its polyglutamyl forms in the Ehrlich ascites tumor versus intestinal epithelial cells after a pulse of drug. The rate of rise, the peak level, and the rate of decline of monoglutamate are comparable in both tissues and, in fact, parallel the changes in the blood level. The critical difference between these tissues is in the high level of polyglutamyl forms of MTX that accumulate in the tumor relative to host tissue so that within 8 to 10 hours after administration of this agent, pharmacologic activity should be expressed primarily in the former. Drug toxicity in epithelial cells is initiated early after MTX administration when the intracellular and extracellular monoglutamyl levels are high with pharmacologic activity diminishing as the drug is cleared (*40*). Likewise, only low levels of MTX polyglutamates are detected in bone marrow cells (*39,41*). Hence, if the host could be protected from the toxic effects of MTX when monoglutamyl levels are high while allowing polyglutamylation to proceed in the tumor, selective cytotoxicity should be expressed in the tumor cells following cessation of protection when the free monoglutamate level is negligible in both tissues.

Based on the clear demonstration of a differential polyglutamylation in host versus tumor tissues, additional studies from this laboratory have confirmed the concept of "early" nucleoside protection in the L1210 murine leukemia model in vivo (*47*). Experiments were undertaken in which mice received MTX pulses at 12.5 to 150 mg/kg/day for 3 consecutive days with or without thymidine and inosine

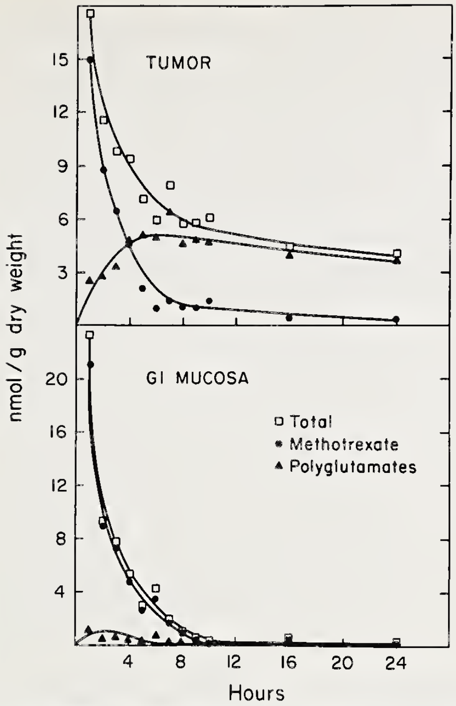


FIGURE 5.—Accumulation and decline of MTX and MTX polyglutamates in Ehrlich ascites tumor cells and the intestinal (GI) mucosa. Tumor-bearing mice were given sc injections of MTX (12 mg/kg) and the drug derivatives were analyzed over the indicated time course (*40*).

protection at 300 mg/kg at 0, 2, 4, and 6 hours following the MTX. Protection increased the MTX dose that produced lethality in 10% of normal mice from 14 to 114 mg/kg/day for 3 days. In tumor-bearing mice, moreover, the maximum dose that could be administered was increased by a factor of 8, although survival was not increased beyond that observed at the optimal dose (12.5 mg/kg/day for 3 days) of MTX alone.

More recent studies in patients at the Medical College of Virginia further confirm the feasibility of "early nucleoside protection" (table 2). In this study, patients were given iv MTX weekly, starting with a dose of 50 mg/m^2 with 50% escalation in dose until mild mucosal or bone marrow toxicity was observed. When toxicity was detected, the MTX dose was held constant and thymidine was infused at 85 mg/m^2/hour to determine the minimum interval of protection necessary to circumvent toxicity. When this interval was established, the MTX dose was once again escalated. In this way, the MTX dose and thymidine infusion interval were modulated in order to define the relationship between the MTX dose and the interval of thymidine infusion necessary to achieve protection and the maximal dose of MTX that could be administered safely by this approach. As observed in table 2, rather high levels of MTX could be administered with acceptable toxicity. Studies are currently underway to evaluate the toxicity of a regimen of 165 mg/m^2 MTX beginning with a 4- to 6-hour interval of thymidine protection. While this study is in an early phase, a number of important observations were made. First, this dose of MTX can be nephrotoxic and this nephrotoxicity occurs very rapidly after administration of drug resulting in a fall in glomerular filtration rate that can be detected by a rise in serum creatinine within 24 hours. Unexpected delayed excretion of the drug rapidly creates a condition in which the duration of thymidine infusion is not sufficiently long to provide an adequate interval of protection. This complication can be obviated by monitoring the serum creatinine and MTX levels 24 hours after the MTX dose is given. If the serum creatinine and/or MTX blood levels are elevated, reinstitution of the thymidine infusion or addition of leucovorin rescue at the 24-hour point should completely circumvent MTX toxicity.

TABLE 2.—Phase 1 study of methotrexate with early nucleoside protection

Patient	Number of treatment cycles[a]	Total weeks of treatment[b]	Highest MTX dose achieved mg/m^2	Number of treatments at highest dose	Total weeks at highest dose	Final interval of thymidine infusion[c] hr	Most severe toxicity[d]	Toxicity expressed after the last dose of MTX[e]
C.C.	11	13	168	6	8	6	M[f]=2+	M[f]=0-1
M.K.	9	9	110	7	7	6	M[f]=2+ WBC=2000[h]	0[g]
B.L.	10	13	250	2	2	10	WBC=2000[h]	0[g]
E.T.	14	30	525	3	6	12	M[f]=2+	0[g]
J.W.	9	9	165	5	5	6	N & V[i]=2+ M[f]=2+	0[g]
M.W.	6	6	250	2	2	4	M[f]=2+ WBC=1600[h]	WBC=1600[h]
R.Y.	8	12	165	2	3	8	M[f]=2+	WBC=1700[h]

[a] The total number of doses of MTX with thymidine protection administered.

[b] The total interval over which MTX was administered. Delays were required to permit toxicity to resolve before the next dose of drug was given.

[c] The final interval of thymidine infusion required at a dose of 85 mg/m^2/hr to prevent or minimize toxicity of the highest dose of MTX indicated.

[d] The most severe toxicity observed at any dose of MTX. Grade 1=transient symptoms; grade 2=vesiculation; grade 3=ulceration.

[e] The toxicity observed with the final dose of MTX administered.

[f] M=mucositis.

[g] 0=no toxicity expressed.

[h] cells/mm^3.

[i] N & V=nausea and emesis.

POTENTIAL ADVANTAGES/DISADVANTAGES OF EARLY THYMIDINE PROTECTION VERSUS LATE LEUCOVORIN RESCUE

It has been clearly established that leucovorin rescue permits the administration of very high doses of MTX in a variety of clinical regimens. This can be done safely if renal function and MTX blood levels are carefully monitored. High-dose MTX with leucovorin rescue can be advantageous in any of the conditions described above, which require high extracellular MTX levels to assure adequate delivery of drug to tumor cells and adequate generation of active polyglutamyl derivatives. The rationale for early nucleoside protection is quite different. First, the dose of MTX administered with nucleoside protection can only be modest since the higher the dose the longer the interval over which high extracellular and intracellular monoglutamate levels are sustained. This would result in a greater duration of risk to the host cells and therefore longer intervals required for nucleoside protection. Furthermore, at high concentrations of MTX sustained over long intervals, it is likely that sufficient levels of polyglutamyl derivatives accumulate in susceptible host cells to produce a sustained pharmacologic effect in these tissues after the monoglutamate is eliminated. Both of these factors would compromise the inherent selectivity of this approach.

Hence, early nucleoside protection could be advantageous in MTX sensitive tumors or under conditions in which there is only moderate drug resistance when a modest increase in drug dose could produce a substantial increase in chemotherapeutic efficacy. This approach might also have utility in the treatment of nonmalignant diseases where low doses of MTX have been used with success. A significant increase in the MTX dose that could be administered safely under these conditions might be associated with dramatic improvements in clinical response.

A potential limitation in leucovorin rescue is the consequence of the progressive buildup of natural folylpolyglutamates within tumor cells that may occur after repetitive exposure to leucovorin. Hence, when leucovorin is administered following MTX, selectivity is enhanced as host cells are rescued and tumor cells are killed. However, if the subsequent dose of MTX is administered at a time when folate levels remain high in the tumor, the antitumor efficacy of the antifolate will be decreased. Studies have demonstrated that prior treatment of tumor cells with leucovorin impairs polyglutamylation of MTX (*48,49*). Furthermore, the loading of cells with reduced folate cofactors could also result in a higher level of interconversion to FH_2 with increased competition between FH_2 and the antifolate at the level of DHFR. This buildup of natural folates within tumor cells could reduce the effectiveness of repetitive treatments with MTX and leucovorin rescue, particularly when the intervals between treatments are brief. Early nucleoside protection on the other hand should preserve selectivity even with repetitive administration because of the rapid consumption of nucleosides. This should leave tumor cells in an essentially unchanged state, ready to metabolize MTX normally and with the same folate cofactor distribution at the time the next dose of MTX is administered.

Another potential advantage to early thymidine protection is the opportunity for repetitive dosing of MTX with the possibility for progressive cell synchronization and repetitive kill of tumor cells at their most vulnerable phase of the cell cycle. For instance, after a pulse of MTX with concurrent nucleoside protection, cell replication is normal during the interval over which MTX polyglutamates accumulate. However, when protection ceases, nucleosides are very rapidly depleted so that tumor cells that have accumulated MTX polyglutamates and are in S phase are killed. Those tumor cells that are out of S phase tend to progress through the cell cycle stopping at the G_1–S interphase or early S phase. Following the second pulse of MTX with nucleoside protection, cells at the G_1–S interphase move into S phase and, along with the cells in early S phase, establish themselves in DNA replication over the interval of protection. With each additional pulse of MTX, this process of synchronization and exposure of S-phase cells to intracellular polyglutamates continues. Hence, not only does this approach have the capacity to enhance selectivity of MTX due to differences in MTX polyglutamylation in tumor versus host tissues but, in addition, it may offer the possibility for enhanced tumor cell kill by progressive synchronization of the tumor cell population with intermittent exposure to drug during the most vulnerable phase of the cell cycle.

REFERENCES

(*1*) Goldin A, Mantel N, Greenhouse SW, et al: Estimation of the antileukemic potency of the antimetabolite aminopterin, administered alone and in combination with citrovorum factor or folic acid. Cancer Res 13:833–850, 1953.

(*2*) Goldin A, Venditti JM, Kline I, et al: Eradication of leukemic cells (L1210) by methotrexate and methotrexate plus citrovorum factor. Nature 212:1548–1550, 1966.

(*3*) Jackson RC, Harrap KR: Studies with a mathematical model of folate metabolism. Arch Biochem Biophys 158:827–841, 1973.

(*4*) White JC: Reversal of methotrexate binding to dihydrofolate reductase. Studies with purified enzyme and computer modelling using network thermodynamics. J Biol Chem 254:10889–10895, 1979.

(*5*) Goldman ID: The mechanism of action of methotrexate. I. Interaction with a low affinity intracellular site for maximum inhibition of deoxyribonucleic and synthesis in L-cell mouse fibroblasts. Mol Pharmacol 10:257–274, 1974.

(*6*) White JC, Goldman ID: Mechanism of action of methotrexate. IV. Free intracellular methotrexate required to suppress dihydrofolate reduction to tetrahydrofolate by Ehrlich ascites tumor cells in vitro. Mol Pharmacol 12:711–719, 1976.

(*7*) White JC, Loftfield S, Goldman ID: The mechanism of action of methotrexate. III. Free intracellular methotrexate is required for maximum suppression of [^{14}C]formate incorporation into nucleic acids and proteins. Mol Pharmacol 11:287–297, 1975.

(*8*) Goldman ID, Lichtenstein NS, Oliverio VT: Carrier-mediated transport at the folic acid analogue, methotrexate, in the L1210 leukemia cell. J Biol Chem 243: 5007–5017, 1968.

(*9*) Goldman ID, Gupta V, White JC, et al: Exchangeable intracellular methotrexate levels in the presence and absence of vincristine at extracellular drug concentrations relevant to those achieved in high-dose methotrexate–folinic acid "rescue" protocols. Cancer Res 36:276–279, 1976.

(*10*) Fry DW, White JC, Goldman ID: Effects of 2,4-dinitrophenol and other metabolic inhibitors on the bidirectional fluxes, net transport, and intracellular binding of methotrexate in Ehrlich ascites tumor cells. Cancer Res 40:3669–3673, 1980.

(*11*) Goldman ID: Transport energetics of the folic acid analogue, methotrexate, in L1210 cells: Enhanced accumulation by metabolic inhibitors. J Biol Chem 244:3779–3785, 1969.

(*12*) Sirotnak FM, Moccio DM, Goutas LJ, et al: Biochemical correlates of responsiveness and collateral sensitivity of some methotrexate-resistant murine tumors to the lipophilic antifolate, metoprine. Cancer Res 42:924–928, 1982.

(*13*) Sirotnak FM, Goutas LJ, Mines LS: Extent of the requirement for folate transport by L1210 cells for growth and leukemogenesis in vivo. Cancer Res 45:4732–4734, 1985.

(*14*) Sirotnak FM, Moccio DM, Goutas LJ, et al: Biochemical correlates of responsiveness and collateral sensitivity of some methotrexate resistant murine tumors to the lipophilic antifolate, metoprine. Cancer Res 42:924–928, 1982.

(*15*) White JC: Predictions of a network thermodynamic computer model relating to the mechanism of methotrexate rescue by 5-formyl-tetrahydrofolate and to the importance of inhibition of thymidylate synthase by methotrexate polyglutamates. *In* Folyl and Antifolyl Polyglutamates. (Goldman ID, Chabner BA, Bertino JR, eds). New York: Plenum Press, 1983, pp 305–327.

(*16*) Matherly LH, Anderson LA, Goldman ID: Role of the cellular oxidation–reduction state in methotrexate binding to dihydrofolate reductase and dissociation induced by reduced folates. Cancer Res 44:2325–2330, 1984.

(*17*) Matherly LH, Barlowe CK, Goldman ID: Antifolate polyglutamylation: A selective determinant of leucovorin rescue. *In* Proceedings of the Second Workshp on Folyl and Antifolyl Polyglutamates (Goldman ID, ed). New York: Praeger, 1985, pp 256–272.

(*18*) Matherly LH, Barlowe CK, Goldman ID: Antifolate polyglutamylation and competitive drug displacement at dihydrofolate reductase as important elements in leucovorin rescue in L1210 cells. Cancer Res 46:588–593, 1986.

(*19*) Matherly LH, Fry DW, Goldman ID: Role of methotrexate polyglutamylation and cellular energy metabolism in inhibition of methotrexate binding to dihydrofolate reductase by 5-formyltetrahydrofolate in Ehrlich ascites tumor cells in vitro. Cancer Res 43:2694–2699, 1983.

(*20*) Schilsky RL, Jolivet J, Bailey BD, et al: Synthesis, binding, and intracellular retention of methotrexate polyglutamates by cultured human breast cancer cells. *In* Folyl and Antifolyl Polyglutamates (Goldman ID, Chabner BA, Bertino JR, eds). New York: Plenum Press, 1983, pp 247–258.

(*21*) Fry DW, Yalowich JC, Goldman ID: Rapid formation of polyglutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high-performance liquid chromatography in Ehrlich ascites tumor cells in vitro. J Biol Chem 257:1890–1896, 1982.

(*22*) Gewirtz DA, White JC, Randolph JK, et al: Formation of methotrexate polyglutamates in rat hepatocytes. Cancer Res 39:2914–2918, 1979.

(*23*) Galivan J: Evidence for the cytotoxic activity of polyglutamate derivatives of methotrexate. Mol Pharmacol 17: 105–110, 1980.

(*24*) Jolivet J, Schilsky RL, Bailey BD, et al: Synthesis, retention, and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. J Clin Invest 70:351–360, 1982.

(*25*) Rosenblatt DS, Whitehead VM, Vera N, et al: Prolonged inhibition of DNA synthesis associated with the accumulation of methotrexate polyglutamates in cultured human cells. Mol Pharmacol 21:718–722, 1978.

(*26*) Borsa J, Whitmore GF: Studies relating to the mode of action of methotrexate. III. Inhibition of thymidylate synthetase in tissue culture cells and cell free systems. Mol Pharmacol 5:318–332, 1969.

(*27*) Allegra CJ, Chabner BA, Drake JC, et al: Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. J Biol Chem 260:9720–9726, 1985.

(*28*) Allegra CJ, Drake JC, Jolivet J, et al: Inhibition of phosphoribosyl aminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. Proc Natl Acad Sci USA 82:4881–4885, 1985.

(*29*) Baggot JE: Inhibition of purified avian liver aminoimidazolecarboxamide ribotide transformylase by polyglutamates of methotrexate and oxidized folates. Fed Proc 42:667, 1983.

(*30*) Allegra CJ, Drake JC, Jolivet J, et al: Inhibition of folate-dependent enzymes by methotrexate polyglutamates. *In* Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates (Goldman ID, ed). New York: Praeger, 1985, pp 348–359.

(*31*) Kisliuk RL, Gaumont Y, Baugh CM: Polyglutamyl derivatives of folate as substrates and inhibitors of thymidylate synthetase. J Biol Chem 249:4100–4103, 1974.

(*32*) Mathews RG, Baugh CM: Interactions of pig liver methylenetetrahydrofolate reductase with methylenetetrahydropteroylpolyglutamate substrates and with dihydropteroylpolyglutamate inhibitors. Biochemistry 19:2040–2045, 1980.

(*33*) Matherly LH, Barlowe CK, Phillips VM, et al: The effects of 4-aminoantifolates on 5-formyltetrahydrofolate metabolism in L1210 cells. A biochemical basis for the selectivity of leucovorin rescue. J Biol Chem 262:710–717, 1987.

(*34*) Allegra CJ, Braham J, Boarman J, et al: Dihydrofolate accumulation: A role in methotrexate inhibition of thymidylate biosynthesis. Proc Am Assoc Cancer Res 28:273, 1987.

(*35*) Moran RG: Depletion of tetrahydrofolate by methotrexate. Proc Am Assoc Cancer Res 23:178, 1982.

(*36*) Wasserman GF, Benkovic PA, Young M, et al: Kinetic relationships between the various activities of the formyl-methenyl-methylene tetrahydrofolate synthetase. Biochemistry 22:1005–1013, 1983.

(*37*) Allegra CJ, Fine RL, Drake JC, et al. The effects of methotrexate on intracellular folate pools in human MCF-7 breast cancer cells. J Biol Chem 261:6478–6485, 1986.

(*38*) Borsa J, Whitmore GF: Cell killing studies on the mode of action of methotrexate in L-cells in vitro. Cancer Res 29:737–744, 1969.

(*39*) Fabre I, Fabre G, Goldman ID: Polyglutamylation, an important element in methotrexate cytotoxicity and selectivity in tumor versus murine granulocytic progenitor cells in vitro. Cancer Res 44:3190–3195, 1984.

(*40*) Fry DW, Anderson LA, Borst M, et al: Analysis of the role of membrane transport and polyglutamylation of methotrexate in gut and the Ehrlich tumor in vivo as factors in drug sensitivity and selectivity. Cancer Res 43:1087–1092, 1983.

(*41*) Koizomi S, Curt GA, Fine RL, et al: Formation of methotrexate polyglutamates in purified myeloid precursor cells from normal human bone marrow. J Clin Invest 75:1008–1014, 1985.

(*42*) Poser RG, Sirotnak FM, Chello PL: Differential synthesis of methotrexate polyglutamates in normal proliferative and neoplastic mouse tissues in vivo. Cancer Res 41:4441–4446, 1981.

(*43*) Semon JH, Grindey GB: Potentiation of the antitumor activity of methotrexate by concurrent infusion of thymidine. Cancer Res 38:2905–2911, 1978.

(*44*) Howell SB, Herbst K, Bass GR, et al: Thymidine requirements for the rescue of patients treated with high-dose methotrexate. Cancer Res 40:1824–1829, 1980.

(*45*) Howell SB, Ensminger WD, Krishan A, et al: Thymidine rescue of high dose methotrexate in humans. Cancer Res 38:325–330, 1978.

(*46*) Schornagel JH, Leyva A, Pinedo HM: Methotrexate with thymidine protection on rescue in advanced head and neck cancer: A phase II study. Cancer Treat Rep 68:543–546, 1984.

(*47*) Eleff M, Franks PE, Wampler GL, et al: Analysis of "early" thymidine/inosine protection as an adjunct to methotrexate therapy. Cancer Treat Rep 69:867–874, 1985.

(*48*) Galivan J, Nimec Z: Effects of folinic acid on hepatoma cells containing methotrexate polyglutamates. Cancer Res 43:551–555, 1983.

(*49*) Nimec Z, Galivan J: Regulatory aspects of the glutamylation of methotrexate in cultured hepatoma cells. Arch Biochem Biophy 226:671–680, 1983.

Determinants of Resistance to Antifolates: Biochemical Phenotypes, Their Frequency of Occurrence and Circumvention[1]

F. M. Sirotnak[2,*]

ABSTRACT—Biochemical alterations associated with acquired resistance of tumor cells to antifolates are diverse and multiple in number. These most often have included both quantitative and qualitative alterations at the level of membrane transport and of the primary intracellular target, dihydrofolate reductase (DHFR). More recent studies suggest determining biochemical alterations at the level of thymidylate synthase activity and 4-aminofolate polyglutamylation. Approaches to the circumvention of acquired antifolate resistance at the level of new drug design are described which incorporate a kinetic analysis of the various biochemical phenotypes and a systematic analysis of their structure–activity relationships. A consideration of the relative frequency of occurrence of individual phenotypes during therapy is also included. This introduces the notion of population genetics in an evaluation of resistance phenomenon and of clinically significant approaches for its circumvention.—NCI Monogr 5:27-35, 1987.

Acquired resistance of tumor cells to folate antagonists emerged (*1*) as a major obstacle to the clinical utility of these agents soon after the initial use (*2*) of methotrexate in childhood leukemia. Now, some 40 years later, acquired resistance to these agents remains a major limitation to their effective clinical utility despite the vast amount of work carried out by numerous investigators [see recent reviews by Bertino (*3*), Albrecht and Biedler (*4*), and Hutchison (*5*)]. Biochemical alterations associated with acquired resistance to folate antagonists are diverse and multiple in number. These most often have included both quantitative and qualitative alterations at the level of membrane transport (*6*) and of the primary intracellular target, DHFR (*3,4*). In addition, other biochemical alterations which may also have a determining role have been observed at the level of thymidylate synthase activity (*7*) and on the polyglutamylation of 4-aminofolates (*8*). One of the most striking genetic phenomena associated with acquired resistance to antifolates has been the amplification of the gene coding for DHFR in tumor cell variants overproducing this enzyme (*9–11*).

In addition to the many excellent reviews already published on acquired resistance to antifolates (*3–5*), the topic of gene amplification as an underlying mechanism for resistance to antifolates and other cytotoxic agents has been thoroughly reviewed by others (*4,11*). In the current article, the author will focus primarily on those aspects of acquired resistance and its circumvention that are most germane to the central theme of this symposium, namely the development of new folate antagonists. Meaningful approaches to the circumvention of acquired antifolate resistance at the level of new drug design would seem to require an incisive kinetic analysis of the various biochemical phenotypes and a systematic analysis of the structure–activity relationships of various antifolates. It also requires a consideration of the relative frequency of occurrence of each phenotype during therapy. The latter aspect raises the issue of the relevance of population genetics in an evaluation of resistance phenomena and of the approaches for its circumvention that have clinical significance.

SOME PRELIMINARY CONSIDERATIONS

The mechanism of cytotoxic action of antifolates now appears to be quite complex, involving multiple biochemical factors at the level of the cytoplasmic membrane and intracellular folate metabolism (*12–15*). The classical folate antagonists, viz, methotrexate, enter tumor cells by a transport mechanism required for the accumulation of the plasma folate, L-5-methyltetrahydrofolate (*6,12,16,17*). Drug entering these cells inhibits DHFR, the primary enzymologic target, blocking synthesis of tetrahydrofolate (THF). Because of the rapid utilization as L-5,10-methylene-THF and conversion to dihydrofolate (DHF) during thymidylate synthesis (*12–16*), THF is depleted and both thymidine and purine synthesis is inhibited. Although 4-aminofolate analogs are potent inhibitors of DHFR [inhibitory constants (K_i) = 10^{-11} to 10^{-12} *M*], accumulation of these analogs at levels substantially beyond the binding equivalence for DHFR are necessary for effective inhibition (*18,19*) because of the accumulation of DHF (*20*) and its competition with these analogs for binding. The folate transport system, in this context, is needed to mediate the required level of intracellular accumulation of these agents.

After entering tumor cells, 4-aminofolates are polyglutamylated at the γ-carboxyl position (*12–15,21,22*). This may

---

ABBREVIATIONS: DHF = dihydrofolate; DHFR = dihydrofolate reductase; FPGS = folylpolyglutamyl synthetase; GAR = glycinamide ribonucleotide; HSR = homogeneously staining regions; K_i = inhibitory constant; K_m = Michaelis-Menten constant; THF = tetrahydrofolate; V_{max} = maximum influx capacity.

---

[1] Supported in part by grants CA08748, CA18856, CA22764 from the National Cancer Institute, CH-26 from the American Cancer Society, and the Elsa U. Pardee Foundation.

[2] Memorial Sloan-Kettering Cancer Center and Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University, New York, NY.

[*] *Reprint requests to:* F. M. Sirotnak, Ph.D., Laboratory for Molecular Therapeutics, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021.

improve their inhibition of DHFR (*12–15,21,22*) and also evoke potent inhibition of secondary targets, i.e., thymidylate synthase, glycinamide ribonucleotide (GAR) transformylase, and 5-amino-imidazole-4-carboxamide ribonucleotide transformylase (*23–25*). Also, work carried out in vitro appears to demonstrate the increased retention of these 4-aminofolate polyglutamates, particularly the higher polyglutamate forms (*26*). The level of accumulation of 4-aminofolates as a polyglutamate appears to be dependent upon the opposing actions of folylpolyglutamyl synthetase (FPGS) (*22*) and folylpolyglutamyl hydrolase (*27*), both of which are present in tumors for the modulation of natural folate levels (*28*).

The extent to which cell death ultimately results from antithymidine or antipurine effects remains controversial. In some studies antithymidine effects were more pronounced (*29,30*). In others, greater antipurine effects were found in rapidly dividing tumor cells than in slowly dividing tumor cells (*31*). The preponderance of either effect was attributed to differences in folate interconversion metabolism, particularly with respect to the disposition of L-5,10-methylene-THF (*32*). It has also been demonstrated that inhibition of thymidylate synthesis leads to accumulation of dUMP and incorporation of uracil in place of thymine in DNA (*33,34*).

TRANSPORT-RELATED METHOTREXATE RESISTANCE

The association of a reduced rate of methotrexate influx with acquired resistance in tumor cells was first reported in a subline of the L5178Y murine leukemia (*35*). A similar observation in the same cell line was made later by other workers (*36*). In other studies, the resistance of L1210 and P288 murine leukemias to methotrexate (*37*) and the resistance of Yoshida rat sarcoma to aminopterin (*38*) were associated with reduced uptake of drug. Unfortunately, an analysis of the data, which would have discriminated among possible kinetic bases for the differences observed, was not carried out in any of these studies. In more recent studies, in which reduced influx of methotrexate was associated with acquired resistance in H35 rat hepatoma cells (*39*) and in another variant of the L5178Y leukemia (*40*), the extent of the reduction was so great that adequate kinetic analysis could not be done.

In the case of methotrexate-resistant L1210 cells, a number of transport-defective phenotypes have been isolated in different laboratories. In earlier work reported from our laboratory on a methotrexate-resistant transport-altered cell line derived in vivo, only a small difference (<50%) was shown for maximum influx capacity (V_{max}) for methotrexate, but a 4-fold difference in influx Michaelis-Menten constant (K_m) was found which accounted for the resistance observed (*41,42*). A more recent report presented evidence for a similar change involving influx K_m for methotrexate in another resistant subline of the L1210 cell derived in vitro (*43*). Related studies by the same group (*44*), using a methotrexate-resistant variant of the W1–L2 human lymphoblastoid leukemia in culture, showed both increased K_m and reduced V_{max} for methotrexate influx. Similar observations were made in the case of a transport-defective, methotrexate-resistant Chinese hamster ovary cell line (*45*). In some of our more recent studies (*46,47*) several different kinetic phenotypes were identified among transport-altered methotrexate-resistant L1210 cell lines derived in vivo. Among these were sublines that exhibited either reduced V_{max} or increased K_m for methotrexate influx. Also, 1 category showed both kinetic changes. In another study of methotrexate-resistant L1210 cells done elsewhere (*48*), both reduced V_{max} and increased K_m for methotrexate influx were observed.

Among the various methotrexate-resistant variants examined (*46*), those exhibiting reduced V_{max} for methotrexate influx also showed reduced influx of L-5-formyl- or L-5-methyl-THF. This result was not unexpected, since both folates share the same system in the plasma membrane mediating entry of these compounds in tumor cells (*6,12,16,17*). Of interest as well was our observation that in variants exhibiting an altered K_m for methotrexate influx, saturability for L-5-formyl- or L-5-methyl-THF was unchanged (*40*). Similar results had been reported earlier from our laboratory for a group of murine tumors with differing degrees of influx saturability for methotrexate which correlated with their therapeutic responsiveness to this agent (*49*). Values for influx K_m for methotrexate varied 4-fold, while those for L-5-formyl-THF, L-5-methyl-THF and folic acid were similar among these cell lines. In light of the kinetic data available from these studies, we would suggest that the mutational alteration in these L1210 cell variants had site-specific effects at the level of a common carrier which discriminated between the binding of the 4-aminofolate and the natural folates.

From the summary presented in table 1, it would appear that a variety of kinetic phenotypes can account for transport-related methotrexate resistance in tumor cells. These include a reduction in the V_{max} of the system affecting the accumulation of both methotrexate and 5-substituted reduced folates and reduced K_m for methotrexate influx which need not affect saturability for natural folate compounds.

It has occasionally been suggested that transport-related methotrexate resistance might be a phenomenon demonstrable only in vitro. Because of greater chemical stability, the growth of tumor cells in culture normally employs folic acid as the sole folate source rather than the circulating plasma folate, L-5-methyl-THF. Also, there appears to be a system in the plasma membrane other than the reduced folate-methotrexate system, at least in L1210 cells (*68*), that will transport this oxidized folate. Therefore, a large defect in transport by the latter system, reducing accumulation of both methotrexate and reduced folates, would be permissive in the presence of folic acid, i.e., it would not compromise the cells' requirement for exogenous folates. However, we (*41,42,45*) and others (*37*) have been able to isolate methotrexate-resistant variants of the L1210 cell and other murine leukemias in vivo that were transport-defective. Thus, it would seem that this form of resistance has both pharmacologic significance and clinical relevance.

In addition to these above findings, we have approached this question in another manner. We have selected for methotrexate-resistant L1210 cells in culture in the presence of folic acid and were able to isolate variant phenotypes with varying degrees of reduction in V_{max} for both methotrexate and 5-substituted reduced folates (*69*). Phenotypes L1210/R1 and R24 (table 2), showing only finite reduction in influx capacity (6- to 16-fold), grew in culture

TABLE 1.—Mechanisms of resistance to folate antagonists

Biochemical property	Phenotype	Drug category	Reference
transport	increased influx K_m (MTX)[a]	analogs	(*41,42,43,46,47*)
	decreased influx V_{max}	analogs	(*46,47,50*)
	increased influx K_m (MTX) and decreased influx V_{max}	analogs	(*44,46,47,48*)
	increased influx V_{max}	lipophiles	(*51*)
DHFR	increased synthesis	analogs, lipophiles	(*10,52–61*)
	decreased drug binding	analogs	(*62–66*)
polyglutamylation	reduced formation of higher polyglutamates(?)	analogs	(*67*)

[a] MTX = methotrexate.

as well as did the parental cells when either folic acid or L-5-formyl-THF was used as the sole source of folate (L-5-formyl-THF was used as a model for the circulating plasma folate, L-5-methyl-THF, which was unstable in culture medium) (*69*). However, phenotype L1210/R25, showing more marked reduction in influx capacity (100-fold), grew only when folic acid was present in the medium. Likewise, when these cell lines were transplanted into mice, only those mice implanted with cultured parental cells or variants L1210/R and L1210/R24, with more finitely altered influx, developed leukemia. However, if mice implanted with L1210/R25 were given repeated doses of folic acid ip at 2.5 mg/kg or above, these animals also developed leukemia. Growth was not restored with either L-5-formyl-THF or L-5-methyl-THF. We also observed among animals which were implanted with a large number of L1210/R25 cells that a few eventually did develop leukemia, but only after a very prolonged delay. We examined the growth in vivo and transport of L-5-methyl-THF in these cells and found that they exhibited characteristics similar to those of L1210/R1 cells (*69*). Thus, it would appear that these cells originated from a revertant with a partially restored capacity for mediated accumulation of folate coenzymes. These results, and the fact that proliferation in vivo could be restored by the administration of folic acid but not by the administration of 5-substituted reduced folates, would suggest that the loss of proliferative potential and leukemogenesis in R25 cells was related only to an impairment of folate coenzyme transport inward. Although there is some variability in V_{max} among various tumors, it would appear that L1210 cells and probably other tumor cells, at least those with similar growth and metabolic potential for folate interconversion, exhibit substantial excess in capacity for folate coenzyme accumulation (*17*). In these cases substantial reduction in influx capacity for L-5-methyl-THF could occur in variants without compromising growth in vivo, and such cells would be highly resistant to classical folate analogs which share this route of entry.

TRANSPORT-RELATED METOPRINE RESISTANCE AND COLLATERAL SENSITIVITY

An interesting finding pertaining to transport-related methotrexate resistance showed that L1210 cell variants with reduced V_{max} for methotrexate became increasingly sensitive (collaterally sensitive) to lipophilic antifols, viz, metoprine (*47*). The insensitivity to methotrexate of the L1210/R1 variant was associated with 3- to 4-fold lower saturability and 6-fold reduced capacity for mediated influx of this analog (table 2). Influx capacity for reduced folates was also reduced 6-fold. In the presence of L-5-formyl-THF as the sole source of folate, substantially less inhibition of growth of the variant was observed with methotrexate, but the variant showed increased sensitivity to metoprine. Similarly, responsiveness in mice to methotrexate was reduced in variant versus parental cells, but responsiveness to metoprine was increased (table 3). It was also found in our own studies (*47*) that the M5076 reticulum cell sarcoma, which was naturally refractive to methotrexate and also exhibited the same deficit in influx capacity for folate coenzymes, was exquisitely sensitive to metoprine. It appears that a relatively low level of accumulation of L-5-methyl-THF (the major circulating plasma folate) in these tumors (L1210/R1

TABLE 2.—Growth characteristics and folate requirements of parental and transport-defective L1210 cells

Cell line[a]	Relative influx capacity, %	Growth in vitro		Leukemogenesis in vivo	
		FA[b]	5-formyl-THF[b]	no FA	plus FA[c]
L1210	100	yes	yes	yes	yes
L1210/R1	15	yes	yes	yes	yes
L1210/R24	6	yes	yes	yes	yes
L1210/R25	1	yes	no	no	yes

[a] Variant cell lines (R1, R24, R25) were derived in culture from a parental cell line already established in culture.

[b] FA = folic acid.

[c] Starting at the time of transplantation, 10 mg/kg was inoculated ip twice daily.

TABLE 3.—Properties of parental and transport-defective, methotrexate-resistant L1210 cells

Cell line	Relative influx capacity, %	Growth inhibition (IC_{50})[a]		Increased life-span	
		MTX,[b] n*M*	metoprine, n*M*	MTX,[c] %	metoprine,[d] %
L1210	100	8.3 ± 1.1	40.2 ± 7	+147 ± 15	+38 ± 3
L1210/R1	15	129 ± 9.2	7.8 ± 1	+9 ± 1	+138 ± 11

[a] In the presence of 5 n*M* L-5-formyl-THF; IC_{50} = concentration of drug to inhibit growth by 50%.

[b] MTX = methotrexate.

[c] 15 mg/kg sc every 2 days for 5 doses.

[d] 28 mg/kg sc every 3 days for 3 doses.

and M5076), in the face of a metoprine-induced blockade at the level of DHFR, probably accounts for their high degree of responsiveness to metoprine and, most likely, other lipophilic antifolates. In support of this notion, we have also isolated metoprine-resistant variants of the L1210 cell with substantially increased influx V_{max} for folate coenzymes that show increased sensitivity to methotrexate (*51*). This phenotype appears to be characterized by an increased rate of synthesis of the putative carrier protein (transporter) for the reduced folate/methotrexate system (*51*).

RESISTANCE RELATED TO INCREASED DHFR ACTIVITY

In very early studies, methotrexate resistance of cultured murine L5178Y and S180 cells was associated with increases in DHFR activity (*70,71*). Similarly, the resistance of 2 of 3 sublines of murine L1210 leukemia derived in vivo was also associated with increased levels of DHFR activity (*72*). The studies with cultured S180 cells documented, as well, the unstable nature of this resistance when highly resistant cells were cultured in the absence of methotrexate (*73*). Studies carried out later (*74–77*) showed that increased activity of this enzyme in this category of resistant variants was due to a quantitative increase in the amount of this enzyme which otherwise was unchanged in its physical and catalytic properties or immunoreactivity (table 1). Other studies provided conclusive evidence that the increases in enzyme content found in these variants were a result of increased synthesis of the enzyme rather than reduced turnover as a consequence of increased stabilization (*52,53*). It was eventually shown that these increases in synthesis of DHFR in these variants was accompanied by elevation in the cellular concentration of DHFR-specific mRNA (*54,55*). These studies employed translation in vitro in a cell-free system.

Very early studies by Littlefield (*56*) raised the notion that DHFR overproduction in methotrexate-resistant variants of BHK cells might be accounted for by the increased cellular content of DHFR structural genes, a phenomenon documented in lower organisms (*57,58*). In studies carried out concurrently at our institution, Biedler and co-workers (*59*) were able to associate in L1210 cell variants elevated DHFR activity and resistance with the disappearance of a specific "subtelocentric" chromosome. In similar studies, this group found resistant variants of Chinese hamster lung cells (DC-3F) that overproduce DHFR, often showing elongated segments on chromosome 2 as well as others (*60,61*). These abnormal segments also stained homogeneously during banding. Since homogeneously staining regions (HSR) were found in the variants exhibiting highest DHFR activity, it was speculated that these regions were functionally involved in the overproduction of this enzyme and were a structural manifestation of an amplification process (*59–61*). Direct evidence using recombinant DNA methodology was eventually obtained elsewhere for DHFR gene amplification in methotrexate-resistant S180 cells (*10*). It was also demonstrated by in situ hybridization that HSR were, in fact, sites of amplified DHFR genes in mouse and Chinese hamster tumor cells (*78,79*). In addition to the HSR, another distinct abnormality that is often seen in DHFR-overproducing murine or human cells is the double-minute chromosome (*80*). These are small, paired extrachromosomal bodies that appear to lack a centromere. In contrast to the relatively stable and irreversible resistance associated with HSR or related abnormally banding regions, resistance associated with double-minutes appears to be unstable. One additional item of interest should also be mentioned which suggests that overproduction of DHFR in tumor cells may result not only from gene amplification. Work carried out by two separate groups on resistant variants of human tumor cells demonstrated a lack of correlation between gene copy number and DHFR level (*81,82*). Therefore, it is quite possible that alteration of DNA sequences other than the DHFR gene may result in an increase in DHFR and specific mRNA by altered gene expression. It is beyond the scope of this article to review additional information pertaining to this important genetic phenomenon. Instead, the reader is referred to a number of excellent reviews on this subject (*4,10,11*).

RESISTANCE RELATED TO STRUCTURALLY ALTERED DHFR

Structurally altered forms of DHFR have been found in a number of tumor cell variants that have acquired resistance to folate antagonists after exposure in cell culture. The first instance of an altered molecular species was documented for a methotrexate-resistant variant of the L4946 murine leukemia (*83*). These workers described differences in certain physical properties of this enzyme and its activation by chloride or sulfhydryl reagents. Other workers described the appearance in L1210 cells of a new electrophoretic form of DHFR during continuous exposure in vivo to methotrexate (*62*). These results were later confirmed during in vitro studies (*43*). The significance, however, of these alterations in variant L4946 and L1210 cells remains unclear with regard to their role in acquired resistance.

A structurally elevated DHFR isolated from methotrexate-resistant Chinese hamster lung cells was studied at our institution by Albrecht and co-workers (*63*). This enzyme species exhibited decreased affinity for methotrexate, but its inhibition by the quinazoline, methasquin, was unchanged. The structural alteration was also reflected as a difference in the number of pH optima and increased lability. Other structurally altered DHFRs with reduced affinity for methotrexate have been described in methotrexate-resistant variants of Chinese hamster ovary cells (*64*), W1–L2 human lymphoblastoid cells (*65*), L5178Y murine leukemia cells (*66*), and 3T6 cells (*84*).

RESISTANCE RELATED TO DECREASED POLYGLUTAMYLATION OR OTHER ENZYMIC ALTERATIONS

Recent evidence derived from our laboratory (*26*) and elsewhere (*85*) has established a close association between the extent of γ-polyglutamylation of folate analogs and their cytotoxicity in cell culture. Other studies have also shown that, in tumor models responding to a specific folate analog, polyglutamylated derivatives of this analog are accumulated to a greater extent in tumor than in normal proliferative tissue where drug-limiting toxicity occurs (*50,67,86*). In recent studies, 2 tumor-cell clones were established in culture from a patient with small-cell lung cancer who relapsed following treatment with a methotrexate-containing regimen of therapy (*87*). These clones exhibited 100-fold increases in resistance to methotrexate in a clonogenic assay compared to another clone derived from another patient with the same disease who had not been treated with methotrexate. The authors did not derive evidence for alterations in transport or DHFR levels in the resistant clones, but found that the ability of these clones to form higher polyglutamates of methotrexate was impaired 1.5- to 4-fold compared with the drug-sensitive clone. Although these differences are unlikely to explain the large differential in methotrexate resistance observed and these workers were unable to obtain direct evidence for an altered FPGS, the results do suggest that defective polyglutamylation may represent a mechanism for resistance to this folate analog.

Similar evidence was also reported by others on methotrexate-resistant cell lines derived from human breast cancer and squamous cell carcinoma (*87,88-90*). Methotrexate-resistant variants of the human breast cancer cell line exhibited reduced transport and decreased accumulation of methotrexate as polyglutamates (*88*). In addition, this line appeared to possess reduced activity of thymidylate synthase. Alterations of transport and thymidylate synthase activity alone did not appear to explain the 100-fold resistance of this variant to methotrexate, suggesting the relevance of the alteration in methotrexate polyglutamate formation to the resistance observed. However, these workers were unable to find evidence for altered FPGS or folypolyglutamyl hydrolase in these variants which might account for this difference. Work carried out with methotrexate-resistant variants of squamous cell carcinoma cells also characterized a variant with reduced transport and reduced accumulation of methotrexate as polyglutamates which appeared to account for the approximate 10- to 12-fold resistance observed (*82,89*). However, no evidence was provided for the mechanism of reduced polyglutamate formation.

RELATIVE FREQUENCY OF RESISTANCE PHENOTYPES

Acquired resistance to chemotherapeutic agents appears, for the most part, to result from random selection of preexisting variant genotypes arising by mutation (*90*). This was shown specifically for antifolates in tumor cell systems when Law and associates (*91*) in 1952 applied the fluctuation test of Luria and Delbruck (*92*) during the development of methotrexate resistance by L1210 cells in vivo. However, gene-amplified antimetabolite resistance in tumor cells may be an important exception to this rule. Recent studies by Schimke and co-workers (*93*) appear to suggest that exposure of cells to antimetabolites randomly will induce amplification of genes through their effects on DNA synthesis. Actual estimates of the frequency of methotrexate-resistant variants of L1210 cell populations in vivo prior to exposure to drug are as high as 1 in 10^4 to 10^5 cells. These values were derived from the earlier studies by Law (*91*), and from other studies by Skipper and co-workers (*94*), both of whom determined the minimum dilution of L1210 cell populations that, when transplanted into mice, would lead to methotrexate resistance following therapeutic challenge. The actual mutation rate, however, is most likely lower, since these estimates for frequency of mutant cells were not carried out with newly cloned sublines.

What determines the frequency in which any one resistance phenotype will appear within a drug-susceptible population? Acquired resistance to antimetabolites is almost always a result of multiple genomic alterations, since their mode of cytotoxic action is usually complex and the number and frequency of these different phenotypes will reflect this complexity and the importance of individual drug-protein interactions (table 4) (*88*). However, the frequency of any specific phenotype will also depend upon constraints at the level of gene expression and their potential for lethality, i.e., permissiveness. For example, the type of genomic alteration of DNA required and the dominance or recessiveness of such an alteration (which might restrict the expression of the alteration) will have a limiting role. As we have seen above and in table 1, a number of specific biochemical phenotypes are associated with resistance to antifolates. However, these appear to occur at very different frequencies within tumor cell populations. A consensus derived from several studies (*3-5*) would appear to suggest that phenotypes characterized by transport alterations or overproduction of DHFR predominate. Moreover, in an actual analysis of the relative frequency of each of these phenotypes clonally derived during initial exposure of L1210 cells to methotrexate in vivo, we have shown that both phenotypes occur with similar frequency (*46*). Also, resistant variants of other phenotypic categories (table 1) were not observed. Although these studies have been expanded since our original report yielding the same conclusion (Sirotnak FM, unpublished observation), the applicability of these findings to other cell types or to any clinical situation must be considered uncertain at this time.

TABLE 4.—Factors limiting the relative frequency of various biochemical phenotypes determining acquired resistance to folate antagonists

1. Complexity of mechanism of cytotoxic action.
2. Relative determining role of individual drug-protein interactions.
3. Constraints at the level of gene expression.
4. Potential for lethality or growth reduction.

CIRCUMVENTION OF ACQUIRED RESISTANCE BY ALTERNATIVE ANTIFOLATE THERAPY

Folate antagonists in use or now under development as antitumor agents comprise a rather large and diverse group

TABLE 5.—Some new folate antagonists currently under study as antitumor agents

Antifolate	Mode of cytotoxic action
1. 4-aminofolates (10-deazaaminopterins)	targeted to DHFR secondarily targeted to folate-dependent enzymes requires transport by the THF-methotrexate system requires polyglutamylation by FPGS
2. lipophilic antifolates (trimetrexate, BW301U)	targeted to DHFR does not require mediated transport or polyglutamylation
3. analogs of folic acid or THF (CB3717), 5, 10-dideazaaminopterin, 5-methyltetrahydrohomofolate	targeted to folate-dependent enzymes requires transport by the THF-methotrexate system or some other system(?) requires polyglutamylation by FPGS
4. inhibitors of FPGS	require transport by the THF-methotrexate system or some other system(?)

Table 6.—Possibilities for alternative antifolate therapy against tumor cells with acquired resistance to methotrexate or lipophilic antifolates

Resistance	Biochemical category	Phenotype	Alternative therapy
MTX[a]	transport (THF/MTX system)	decreased V_{max}	lipophilic antifolate
		increased K_m	lipophile, new 4-amino-folates, analogs of FA[b] or THF
lipophilic agent	transport	increased V_{max}	MTX or new classical analogs
MTX, lipophiles	DHFR	increased level	FA or THF analogs or inhibitors of FPGS (?)
MTX	DHFR	decreased affinity	lipophilic agent (?) FA or THF analogs

[a] MTX = methotrexate.
[b] FA = folic acid.

of structures. Among the classical antifols, methotrexate remains the only agent currently in clinical use. Among the series of newer 4-aminofolates under development, the 10-deazaaminopterins (*50,67,95*), in particular 10-ethyl-10-deazaaminopterin, appear to show promise for broadening clinical utility of this category of agent. Like methotrexate, these new 4-aminofolates (table 5) are targeted to DHFR, and their entry into tumor cells requires mediation by the high-affinity, reduced folate transport system. Conversion to polyglutamates by FPGS appears necessary for effective cytotoxic action (*50*). The improved therapeutic index of 10-ethyl-10-deazaaminopterin can be accounted for by more selective membrane transport and polyglutamylation than methotrexate, favoring greater accumulation of the cytotoxic metabolites in most tumor cells (*15,48*). Another group of folate analogs under more recent development are those targeted to folate-dependent biosynthetic enzymes (table 5). These include the N^{10}-propargyl-quinazoline folic acid analog, CB3717 (*96*), and the THF analog, 5,10-dideaza-THF (*97,98*). The former appears to be targeted to thymidylate synthase, while the latter seems to be targeted to GAR transformylase. Another member of this series, L-5-methyltetrahydrohomofolate, has been under study for some time, but its site of inhibition has not been determined (*99*). Like the 4-aminofolates, all three structures are highly charged anions and their entry into tumor cells would be expected to require mediation by one of two mechanisms transporting folate compounds shown to exist in tumor cells (*68*), including the high-affinity, reduced folate methotrexate transport system. In addition, based on findings showing that these structures are good substrates for FPGS (*8,100*), their inhibitory action on folate-dependent enzymes is most likely in the form of a polyglutamate.

A large group of lipophilic antifolates targeted to DHFR have also been under study (table 5). The most intensively studied of this group incorporate the 2,4-diaminopyrimidine as a 5-arylpyrimidine (metoprine), as a pyridopyrimidine (BW301), or as an unconjugated quinazoline derivative (trimetrexate) (*101*). The latter 2 structures are now under clinical development (discussed elsewhere in this volume). Finally, a new class of antifolates is now under development as inhibitors of FPGS (table 5). Most of these currently are classified as classical folate analogs (*22*). As such, they would be expected to require transport inward by a mediated system.

In view of the information already available on the various biochemical phenotypes associated with acquired resistance to antifolates (see above), an examination of the diversity in properties of these various agents suggests a number of approaches to circumventing this resistance (table 6). Transport-related resistance appears to arise by either 1, or both, of 2 phenotypic alterations of the reduced folate–methotrexate transport system: increased K_m reducing saturability of this system or reduced V_{max} for influx. Both alterations would be circumvented by an effective lipophilic antifolate. Also, in the latter case, there is now considerable evidence for expecting collateral sensitivity to these lipophilic agents (*47*). For methotrexate resistance due only to decreased saturability, there is reason to believe that this alteration is quite specific for 4-aminofolates with an alkyl substitution at N^{10} (*15*). Thus, 10-deazaaminopterins, which incorporate a carbon at position 10, and other analogs of folic acid and THF would most likely be unaffected in their mediated entry by this type of resistance. Also, in the case of the analogs of folic acid and THF, a high-capacity system transporting folic acid inward may offer an effective alternative route for entry in resistant cells in both transport categories (*68*). Finally, variants with resistance to lipophilic antifols mediated through an increase in L-5-methyl-THF influx exhibit collateral sensitivity to 4-aminofolates.

Tumor cells resistant to either methotrexate, 4-aminofolates or lipophilic antifolates by virtue of an elevated level of DHFR would be good candidates for therapy with analogs of THF that are potent inhibitors of folate-dependent enzymes, providing that these analogs do not derive a portion of their activity by inhibition of DHFR. Similar potential exists for inhibitors of FPGS, assuming that these can be found and that they are not undueley restricted by transport barriers. In the case of methotrexate-resistant variants exhibiting an altered DHFR, there is some evidence to suggest that some lipophilic antifols may retain a good measure of inhibitory activity against this enzyme (*102*).

REFERENCES

(*1*) Law LW: Response of a resistant variant of leukemia cells to an antagonist of pteroylglutamic acid. Proc Soc Exp Biol Med 74:599–602, 1951.

(*2*) Farber S, Diamond LK, Mercer RD, et al: Temporary remissions in acute leukemia in children produced by folic acid antagonist 4-amethopteroylglutamic acid (aminopterin). N Engl J Med 238:787–793, 1948.

(*3*) Bertino JR, Carman MD, Weiner HL, et al: Gene amplification and altered enzymes as mechanisms for development of drug resistance. Cancer Treat Rep 67:901–904, 1983.

(*4*) Albrecht AM, Biedler JI: Acquired resistance of tumor cells to folate antagonists. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, ed), vol 1, New York: Academic Press, 1984, pp 317–347.

(*5*) Hutchison DJ: Modes of acquiring resistance to antineoplastic agents. *In* Experimental and Clinical Chemotherapy (Kuemmerle HP, ed), vol 3. Stuttgart: Thieme, 1983, pp 368–383.

(*6*) Dembo M, Sirotnak FM: Membrane transport of folate compounds in mammalian cells *In* Folate Antagonists as Therapeutic Agents, (Sirotnak FM, ed), vol 1. New York: Academic Press, 1984, pp 113–214.

(*7*) White JC, Goldman ID: Methotrexate resistance in an L1210 cell line resulting from increased dihydrofolate reductase, decreased thymidylate synthetase activity and normal membrane transport. J Biol Chem 256:5722–5727, 1981.

(*8*) Curt G, Carney D, Jolivet J, et al: Defective methotrexate (MTX G_1) polyglutamylation: A mechanism of drug resistance in human small cell lung cancer (SCLC). Proc Am Assoc Cancer Res, 24:283, 1983.

(*9*) Johnston RN, Beverley SM, Schimke RT: Rapid spontaneous dihydrofolate reductase gene amplification shown by fluorescence-activated cell sorting. Proc Natl Acad Sci USA, 80:3711–3715, 1983.

(*10*) Schimke RT, Kaufman RJ, Nunberg JH, et al: Studies on the amplification of dihydrofolate reductase genes in methotrexate-resistant cultured mouse cells. Cold Spring Harbor Symp Quant Biol 61:1297–1303, 1979.

(*11*) Schimke RT: Gene amplification, drug resistance and cancer. Cancer Res 44:1735–1742, 1984.

(*12*) Sirotnak FM: Correlates of folate analog transport, pharmacokinetics and selective antitumor action. Pharmacol Therapeut 8:71–103, 1980.

(*13*) Chabner BA: Methotrexate. *In* Pharmacologic Principles of Cancer Treatment (Chabner BA, ed). Philadelphia: W. B. Saunders, 1982, pp 229–255.

(*14*) Jackson RC, Grindey GB: The biochemical basis for methotrexate cytotoxicity. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, ed) vol 1. New York: Academic Press, 1984, pp 290–316.

(*15*) Sirotnak FM, DeGraw JI: Selective antitumor action of folate analogs *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, ed). New York: Academic Press, 1984, pp 43–95.

(*16*) Goldman ID: The characteristics of membrane transport of amethopterin and the naturally occurring folates. Ann NY Acad Sci 186:400–422, 1971.

(*17*) Sirotnak FM: Obligate genetic expression in tumor cells of a fetal membrane property mediating "folate" transport: Biological significance and implications for improved therapy of human cancer. Cancer Res 45:3992–4000, 1985.

(*18*) Sirotnak FM, Donsbach RC: The intracellular concentration-dependence of antifolate inhibition of DNA synthesis in L1210 leukemia cells. Cancer Res 34:3332–3340, 1974.

(*19*) White JC, Goldman ID: Mechanism of action of methotrexate. IV. Free intracellular methotrexate required to suppress dihydrofolate reduction to tetrahydrofolate by Ehrlich ascites tumor cells in vitro. Mol Pharmacol 12:711–719, 1976.

(*20*) Jackson RC, Harrap KR: Studies with a mathematical model of folate metabolism. Arch Biochem Biophys 158:827–841, 1973.

(*21*) Goldman ID, Matherly LH, Fabre G: Methotrexate, aminopterin and 7-hydroxymethotrexate: recent concepts on the cellular pharmacology of 4-amino-antifolates. Prog Cancer Res Ther 28:19–40, 1983.

(*22*) McGuire JJ, Mini E, Hsieh P, et al: Folyl-polyglutamate synthetase: relation to methotrexate action and as a target for new drug development. Development of target-oriented anticancer drugs. Prog Cancer Res Ther 28: 97–106, 1983.

(*23*) Kisliuk RL, Gaumont Y, Baugh CM, et al: Polyglutamyl derivatives of folate as substrates and inhibitors of thymidylate synthetase. J Biol Chem 249:4100–4103, 1974.

(*24*) Baggott JE: Inhibition of purified avian liver aminoimidazolecarboxamide ribotide transformylase by polyglutamates of methotrexate and oxidized folates. Fed Proc 42:667, 1983.

(*25*) Allegra CJ, Drake JC, Jolivet J, et al: Inhibition of the folate-dependent enzymes of de novo purine synthesis and folate interconversion by methotrexate polyglutamates. Proc Am Assoc Cancer Res 25:6, 1984.

(*26*) Samuels LL: Similar differential for total polyglutamylation and cytotoxicity among various folate analogues in human and murine tumor cells in vitro. Cancer Res 45:1448–1495, 1985.

(*27*) Samuels LL, Goutas LJ, Priest DG, et al: Hydrolytic cleavage of methotrexate-gamma-polyglutamates by folylpolyglutamylhydrolase from various tumors and normal tissues of the mouse. Cancer Res 46:2230–2235, 1986.

(*28*) Covey JM: Polyglutamate derivatives of folic acid and coenzymes and methotrexate. Life Sci 26:665–678, 1980.

(*29*) Zaharko DS, Fung W-P, Yang K-H: Relative biochemical aspects of low and high doses of methotrexate in mice. Cancer Res 37:1602–1607, 1977.

(*30*) Donehower RC, Allegra JC, Lippman ME, et al: Combined effects of methotrexate and 5-fluoropyrimidine on human breast cancer cells in serum free medium. Eur J Cancer 16:655–661, 1980.

(*31*) Jackson RC, Weber G: Enzyme pattern directed chemotherapy: the effects of combinations of methotrexate, 5-fluorodeoxoyuridine and thymidine cells in rat hepatoma in vitro. Biochem Pharmacol 25:2613–2618, 1976.

(*32*) Tattersall MH, Jackson RC, Jackson ST, et al: Factors determining cell sensitivity to methotrexate: Studies of folate and deoxyribonucleotide triphosphate pools in five mammalian cell lines. Eur J Cancer 10:819–826, 1974.

(*33*) Fridland A: Effect of methotrexate on deoxynucleotide pools and DNA synthesis in human lymphocyte cells. Cancer Res 34:1883–1888, 1974.

(*34*) Goulian M, Bleile B, Tseng BY: Methotrexate-induced misincorporation of uracil into DNA. Proc Natl Acad Sci USA 77:1956–1960, 1980.

(*35*) Fisher GA: Defective transport of amethopterin (methotrexate) as a mechanism of resistance to the antimetabolites in L5178Y leukemia cells. Biochem Pharmacol 11: 1233–1234, 1962.

(*36*) Harrap KR, Hill BT, Furness ME, et al: Sites of action of amethopterin: intrinsic and acquired drug resistance. Ann NY Acad Sci 186:317–324, 1971.

(*37*) Kessel D, Hall TC, Roberts D, et al: Uptake as a determinant of methotrexate response in mouse leukemias. Science 150:752–754, 1965.

(*38*) Braganca BM, Divekar AY, Vaidya NR: Defective transport of aminopterin in relation to development of resistance in Yoshida sarcoma cells. Biochem Biophys Acta 135:937–946, 1967.

(*39*) Galivan J: Transport and metabolism of methotrexate in normal and resistant cultured rat hepatoma cells. Cancer Res 39:735–743, 1979.

(*40*) Hill BT, Bailey BD, White JC, et al: Characteristics of transport of 4-aminofolates and folate compounds by two lines of L5178Y lymphoblasts, one with impaired transport of methotrexate. Cancer Res 39:2440–2446, 1979.

(*41*) Sirotnak FM, Kurita S, Sargent MG, et al: Sequential biochemical alteration to antifolate resistance in L1210 leukemia. Nature 216:1236–1237, 1967.

(*42*) Sirotnak FM, Kurita S, Hutchison DJ: On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukemia. Cancer Res 28:75–80, 1968.

(*43*) Jackson RC, Niethammer D, Huennekens FM: Enzymatic and transport mechanisms of amethopterin resistance in L1210 mouse leukemia cells. Cancer Biochem Biophys 1:151–155, 1975.

(*44*) Niethammer D, Jackson RC: Changes in molecular properties associated with the development of resistance against methotrexate in human lymphoblastoid cells. Eur J Cancer 11:845–854, 1975.

(*45*) Flintoff WF, Nagaines CR: Transport of methotrexate in Chinese hamster ovary cells: a mutant defective in methotrexate uptake and cell binding. Arch Biochem Biophys 223:433–440, 1983.

(*46*) Sirotnak FM, Moccio DM, Kelleher LG, et al: Relative frequency and kinetic properties of transport defective phenotypes among methotrexate resistant L1210 clonal cell lines derived in vivo. Cancer Res 41:4447–4452, 1981.

(*47*) Sirotnak FM, Moccio DM, Goutas LJ, et al: Biochemical correlates of responsiveness and collateral sensitivity of some methotrexate-resistant murine tumors to the lipophilic antifolate, metoprine. Cancer Res 42:924–928, 1982.

(*48*) McCormick JI, Susten SS, Freisheim JH: Characteristics of the methotrexate transport defect in a resistant L1210 lymphoma cell line. Arch Biochem Biophys 213:311–318, 1981.

(*49*) Sirotnak FM, Donsbach RC: Kinetic correlates of methotrexate transport and therapeutic responsiveness in murine tumors. Cancer Res 36:1151–1158, 1976.

(*50*) Sirotnak FM, DeGraw JI, Moccio DM, et al: New folate analogs of the 10-deazaaminopterin series. Basis for structural design and biochemical and pharmacologic properties. Cancer Chemother Pharmacol 12:18–25, 1984.

(*51*) Sirotnak FM, Moccio DM, Yang C-H: A novel class of genetic variants of the L1210 cell up-regulated for folate analogue transport inward. Isolation, characterization, and degree of metabolic instability of the system. J Biol Chem 259:13139–13144, 1984.

(*52*) Alt FW, Kellems RE, Schimke RT: Synthesis and degradation of folate reductase in sensitive and methotrexate resistant lines of S180 cells. J Biol Chem 251: 3063–3074, 1976.

(*53*) Hänggi VM, Littlefield JW: Altered regulation of the rate of synthesis of dihydrofolate reductase in methotrexate-resistant hamster cells. J Biol Chem 251:3075–3080, 1976.

(*54*) Chang SE, Littlefield JW: Messenger RNA levels in methotrexate resistant BHK cells. Cell 7:391–396, 1976.

(*55*) Kellems RE, Alt FW, Schimke RT: Regulation of folate reductase synthesis in sensitive and methotrexate resistant Sarcoma 180 cells. J Biol Chem 251:6987–6993, 1976.

(*56*) Littlefield JW: Hybridization of hamster cells with high and low folate reductase activity. Proc Natl Acad Sci USA 62:88–95, 1969.

(*57*) Anderson RP, Miller CG, Roth JR: Tandem duplications of the histidine operon observed following general transduction in *Salmonella typimurium*. J Mol Biol 105:201–209, 1976–49:727–764, 1980.

(*58*) Anderson RP, Roth JR: Tandem genetic duplications in phage and bacteria. Annu Rev Microbiol 31:473–505, 1977.

(*59*) Biedler JL, Albrecht AM, Hutchison DM et al: Drug response, dihydrofolate reductase and cytogenetics of amethopterin-resistant Chinese hamster cells in vitro. Cancer Res 32:153–161, 1972.

(*60*) Biedler JL, Spengler BA: A metaphase chromosome anomaly: Association with drug resistance and cell specific products. Science 191:185–187, 1976.

(*61*) Biedler JL, Spengler BA: Quantitative relationship between a chromosome abnormality (HSR) and antifolate resistance associated with enzyme overproduction. J Cell Biol 70 (Part 2):177a, 1976.

(*62*) Harding NGL, Martelli MF, Huennekens FM: Amethopterin-induced changes in the multiple forms of dihydrofolate reductase from L1210 cells. Arch Biochem Biophys 137:295–296, 1970.

(*63*) Albrecht AM, Biedler JL, Hutchison DJ: Two different species of dihydrofolate reductase in mammalian cells differentially resistant to amethopterin and methasquin. Cancer Res 32:1539–1546, 1972.

(*64*) Flintoff WF, Davidson SV, Siminovitch L: Isolation and characterization of three methotrexate-resistant phenotypes from Chinese hamster ovary cells. Somatic Cell Mol Genet 2:245–261, 1976.

(*65*) Jackson RA, Niethammer D: Acquired methotrexate resistance in lymphoblasts resulting from altered kinetic properties of dihydrofolate reductase. Eur J Cancer 13:567–575, 1977.

(*66*) Goldie JH, Dedhar S, Krystal G: A methotrexate-insensitive variant of folate reductase present in two lines of methotrexate-resistant L5178Y cells. Eur J Cancer 16:1539–1546, 1980.

(*67*) Sirotnak FM, DeGraw JI, Schmid FA, et al: New folate analogs of the 10-deaza-aminopterin series. Further evidence for markedly increased antitumor efficacy compared with methotrexate in ascitic and solid murine tumor models. Cancer Chemother Pharmacol 12:26–30, 1984.

(*68*) Yang C-H, Dembo M, Sirotnak FM: Relationships between carrier-mediated transport of folate compounds by L1210 leukemia cells. Evidence for multiplicity of entry routes with different kinetic properties expressed in plasma membrane vesicles. J Membr Biol 25:11–20, 1983.

(*69*) Sirotnak FM, Goutas LJ, Mines LS: Extent of the requirement for folate transport by L1210 cells for growth and leukemogenesis in vivo. Cancer Res 45: 4732–4734, 1985.

(*70*) Fischer GA: Increased levels of folic acid reductase as a mechanism of resistance to amethopterin in leukemia cells. Biochem Pharmacol 11:1233–1237, 1961.

(*71*) Werkheish WC: Specific bindings of 4-amino folic analogues by folic reductase. J Biol Chem 236:888–893, 1960.

(*72*) Friedkin M, Crawford EJ, Goldin A: The association of increased dihydrofolate reductase with amethopterin resistance in mouse leukemia. Cancer Res 22:600–606, 1962.

(*73*) Hakala MT, Zakrzewski SF, Nichol CA: Relation of folic acid reductase to amethopterin resistance in cultured mammalian cells. J Biol Chem 251:3075–3080, 1961.

(*74*) Kashket ER, Crawford EJ, Friedkin M, et al: On the similarity of dihydrofolate reductase from amethopterin-sensitive and amethopterin-resistant mouse leukemia (L1210) cells. Biochemistry 3:1928–1931, 1964.

(*75*) Zakrzewski SF, Hakala MT, Nichol CA: Purification and properties of folate reductase, the cellular receptor of amethopterin. Mol Pharmacol 2:423–431, 1966.

(*76*) Perkins JP, Hillcoat BL, Bertino JR: Dihydrofolate reductase from a resistant subline of the L1210 lymphoma. J Biol Chem 242:4471–4776, 1967.

(*77*) Raunio RP, Hakala MT: Comparison of folate reductases of Sarcoma 180 cells, sensitive and resistant to amethopterin. Mol Pharmacol 3:279–283, 1967.

(*78*) Nunberg JH, Kaufman RJ, Schimke RT, et al: Amplified dihydrofolate reductase are localized to a homogeneously staining region of a single chromosome in a methotrexate resistant Chinese hamster ovary cell line. Proc Natl Acad Sci USA 75:5553–5556, 1978.

(*79*) Dolnick BJ, Bereson RJ, Bertino JR, et al: Correlation of dihydrofolate reductase elevation with gene amplification in a homogenously staining chromosomal region in L5178Y cells. J Cell Biol 83:394–402, 1979.

(*80*) Kaufman RJ, Brown PC, Schimke RT: Amplified dihydrofolate reductase genes in unstable methotrexate-resistance cells are associated with double minute chromosomes. Proc Natl Acad Sci USA 76:5669–5673, 1979.

(*81*) Bouchard J, Bastow KF, Starnes MC, et al: Characterization of dihydrofolate reductase-related DNA and RNA from human KB cell subclones containing different amount of enzyme. 45:1717–1722, 1985.

(*82*) Frei E III, Rosowsky A, Wright JE, et al: Development of methotrexate resistance in a human squamous cell carcinoma of the head and neck in culture. Proc Natl Acad Sci USA 81:2873–2871, 1984.

(*83*) Blumenthal G, Greenberg D: Evidence for two molecular species of dihydrofolate reductase in amethopterin resistant and sensitive cells of the mouse leukemia L4946. Oncology 24:223–229, 1970.

(*84*) Haber DA, Schimke RT: Unstable amplification of an altered dihydrofolate reductase gene associated with double-minute chromosomes. Cell 26:355–362, 1981.

(*85*) Fry DW, Anderson LA, Borst M, et al: Analysis of the role of membrane transport and polyglutamylation of methotrexate in gut and Ehrlich tumor in vivo as factors in drug sensitivity and selectivity. Cancer Res 43:1087–1092, 1983.

(*86*) Poser RG, Sirotnak FM, Chello PL: Differential synthesis of methotrexate polyglutamates in normal proliferative and neoplastic mouse tissues in vivo. Cancer Res 41:4441–4446, 1981.

(*87*) Curt G, Carney D, Jolivet J, et al: Defective methotrexate (MTXG_1) polyglutamation: A mechanism of drug resistance in human small cell cancer (SCLC) Proc Am Assoc Cancer Res 24:283, 1983.

(*88*) Cowan KH, Jolivet J: A methotrexate-resistant human breast cancer cell line with multiple defects, including diminished formation of methotrexate polyglutamates. J Biol Chem 259:10793–10800, 1984.

(*89*) Rosowsky A, Wright JE, Cucchi CA: Phenotypic heterogeneity in cultured human head and neck squamous cell carcinoma lines with low-level methotrexate resistance. Cancer Res 45:6205–6212, 1985.

(*90*) Sirotnak FM: Chromosomal mutation to drug resistance. *In* Antibiotics and Chemotherapy (Hahn FE, ed), vol 20. Basel: S. Karger, 1976, pp 67–80.

(*91*) Law LW: Origin of the resistance of leukemic cells to folic acid antagonists. Nature 169:628–629, 1952.

(*92*) Luria SE, Delbruck M: Mutations in bacteria from virus sensitivity to virus resistance. Genetics 28:491–511, 1943.

(*93*) Hill AB, Schimke RT: Increased gene amplification in L5178Y mouse lymphoma cells with hydroxyurea-induced chromosomal alterations. Cancer Res 45:5050–5057, 1985.

(*94*) Skipper HE, Heidelberger C, Welch AD: Some biochemical problems of cancer chemotherapy. Nature 179:1159–1162, 1957.

(*95*) Schmid FA, Sirotnak FM, Otter GM, et al: New folate analogs of the 10-deaza-aminopterin series. Markedly increased antitumor activity of the 10-ethyl analog compared to the parent compound and methotrexate against some human tumor xenografts in nude mice. Cancer Treat Rep 69:551–553, 1985.

(*96*) Jones TR, Calvert AH, Jackman AL, et al: A potent antitumor quinazoline inhibitor of thymidylate synthetase: synthesis, biological properties and therapeutic results in mice. Eur J Cancer 17:11–19, 1981.

(*97*) Taylor EC, Harrington PJ, Fletcher SR, et al: Synthesis of the antileukemic agents 5,10-dideazaaminopterin and 5,10-dideaza-5,6,7,8-tetrahydroaminopterin. J Med Chem 28:914–921, 1985.

(*98*) Moran RG, Taylor EC, Beardsley GP: 5,10-Dideaza-5,6,7,8-tetrahydrofolic acid. A potent antifolate inhibitor to de novo purine synthesis. Proc Am Assoc Cancer Res 26:231, 1985.

(*99*) Kisliuk RL: Homofolates and other 2-amino-oxyfolates. *In* New Approaches to the Design of Neoplastic Agents (Bardos TJ, Kalman TI, eds). New York: Elsevier, 1982, pp 201–214.

(*100*) Moran RG, Colman PD, Rosowsky A, et al: Structural features of 4-aminofolates required for substrate activity with mammalian folylpolyglutamates synthetase. Mol Pharmacol 27:156–166, 1985.

(*101*) Werbel LM: Design and synthesis of lipophilic antifols as anticancer agents. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, ed), vol 1. New York: Academic Press, 1984, pp 261–284.

(*102*) Sirotnak FM: Two different effects of mutation on the binding of folate antagonists by a microbial dihydrofolate reductase. Biochim Biophys Acta 302:13–23, 1973.

Folate and Antifolate Cell Binding and Accumulation at Physiologic Folate Concentrations[1]

Barton A. Kamen[2,3,4,*]

ABSTRACT—**Cellular transport of folate has been shown to be via either a carrier-mediated or -facilitated diffusion process. The transport constant (K_t) values have ranged from 1 to 20 μM in vitro. These studies have generally been done on cells grown in high concentrations of folic acid (10^{-6} to 10^{-5} M). When cells were grown in folate-depleted medium for 72 hours, growth was nearly equivalent to that observed in complete medium, but a significant quantity of a high-affinity membrane receptor for folate was detected. The dissociation constants for folic acid, 5-methyltetrahydrofolate and methotrexate were 0.4, 4, and 18 n*M*, respectively. Incubation of folate-depleted cells for 3 hours at 10^{-8} M methyltetrahydrofolate restored the intracellular folate content to values equivalent to those found in cells grown in folate-complete medium and decreased membrane binding by >95%. Binding of 5-methyltetrahydrofolate was inhibited by methotrexate, folic acid, and 5-formyltetrahydrofolate, but not by glutamic acid, pteroic acid, or *p*-aminobenzoylglutamic acid. Antisera against a folate-binding protein (FBP) blocked cell binding of folate and purified FBP also blocks cell binding of folate. These results suggest that there is a receptor-mediated uptake of folate, functional at physiologic concentrations of plasma folate. This receptor could potentially be a new membrane target for antifol, antineoplastic agents.—NCI Monogr 5:37–39, 1987.**

The transport of folates and antifolates by both prokaryotes and eukaryote cells has been extensively studied in vitro (*1*). The process(es) involved include a carrier-mediated and/or -facilitated diffusion process for influx and a separate energy-dependent, carrier-mediated efflux. In addition, in L1210 cells, folate and methotrexate transport has been shown to be via an anion transport system (*2*). K_t values for folates and antifolates such as methotrexate are generally 1 to 20 μM. Sirotnak, in a recent review (*1*), has suggested that the transport system, as described, is inefficient compared to transport systems for other important cellular components such as nucleosides. Reasons for this apparent inefficiency are unknown.

Our laboratory has also been concerned about the apparent inefficiency of folate transport with measured K_t values of >1 μM. This is because in man, plasma folate is only 5 to 10 n*M*, but tissue content of folate ranges from 3 to 20 nmol/g (i.e., about 3 to 20 μM). This implies that tissues in situ can effectively concentrate folate approximately 100 to 1000 times greater than the plasma content and, as a corollary, it suggests that there is an efficient transport mechanism for folate (and possibly antifolates) functioning at nanomolar rather than micromolar concentration.

Since most transport studies in vitro have been done on cells growing in high folic acid (10^{-6} to 10^{-5} M) and cells in vitro have a higher folate content than similar cells in vivo, we reasoned that a high-affinity receptor mediating folate transport at physiologic concentrations would not be detected. Therefore, we studied folate and methotrexate transport in cells that had been grown for 72 hours in vitro in folate-deficient medium with intracellular folate pools which had been decreased by 75%.

MA104 (monkey kidney epithelial cells) were chosen because they are already adapted for growth in M199 containing 5% fetal calf serum (vol/vol), a low-folate medium (approximately 3×10^{-8} M). In comparison, more commonly studied cells such as L1210 and L5178Y murine leukemia are routinely grown in RPMI or Dulbecco's modified Eagles medium that contain 100 to 1000 times more folate.

Folate-depleted medium (<0.4 n*M*) was prepared by formulating RPMI 1640 without folic acid and adding 5% (vol/vol) charcoal-treated serum (*3*). After growth for 72 hours in this medium, the binding of radiolabeled folates as a function of extracellular folate concentration was measured at 4°C. As seen in figure 1, the cells exhibited a saturable binding of approximately 1 pmol ligand/10^6 cells for folic acid, 5-methyltetrahydrofolic acid, and methotrexate. One-half maximum binding of each analog was achieved at 0.4, 4, and 18 n*M*, respectively. The specificity of the receptor was confirmed by showing that folic acid, methotrexate, and leucovorin (5-formyltetrahydrofolate) but not glutamic acid, pteroic acid, or *p*-aminobenzoyl glutamic acid could block binding of [^{3}H]5-methyltetrahydrofolate.

The effect of the total cellular folate content on binding of [^{3}H]5-methyltetrahydrofolate by MA104 cells at 4°C is shown in figure 2. Below a cell folate content of 1 pmol/10^6 cells there was a rapid increase in folate binding. Cells grown longer than 96 hours, in order to reduce the folate content below 0.5 pmol/10^6 cells, demonstrated decreased viability and growth as well as decreased binding of folate.

ABBREVIATIONS: FBP = folate-binding protein; K_t = transport constant.

---

[1] Supported by American Cancer Society grant CH-228.

[2] Departments of Pediatrics and Pharmacology, University of Texas Southwestern Medical School, Dallas.

[3] Scholar of the Leukemia Society of America.

[4] I thank Antonieta Capdevila for technical assistance and Dawn Miller for secretarial assistance.

[*] *Reprint requests to:* Barton A. Kamen, M.D., Ph.D., Departments of Pediatrics and Pharmacology, University of Texas Southwestern Medical School, Dallas, TX 75235.

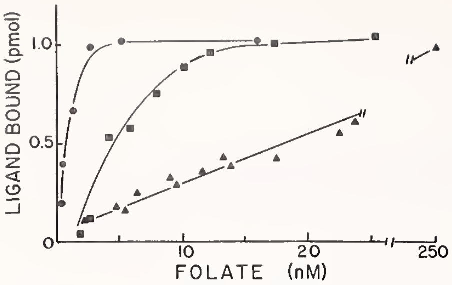


FIGURE 1.—Binding of radiolabeled folates by folate-depleted MA104 cells. MA104 cells (2×10^5) were plated in a T-25 flask. After 6 to 8 hr in folate-complete medium (M199 containing 5% serum), the medium was replaced by RPMI 1640 without folic acid and containing 5% serum that was folate-free, and the cells grown for 3 days. The binding of [^{3}H]folic acid (●), [^{3}H]5-methyltetrahydrofolate (■) and [^{3}H]methotrexate (▲) at 4°C was then determined. The cells were washed with phosphate-buffered saline and removed from the plates for counting. All values are minus a background value determined by measuring radioactivity bound to the cells in the presence of a 1000-fold excess of unlabeled ligand. Reprinted with permission from Proc Natl Acad Sci USA 83:5983–5987, 1986.

Therefore, no studies were done on cells grown longer than 4 days in folate-deficient medium.

MA104 cells grown in folate-complete medium contained 3 to 4 pmol folate/10^6 cells. Cellular accumulation at 37°C and binding at 4°C of 5-methyltetrahydrofolate by folate-depleted and -replete cells are shown in figure 3. As observed, after 3 hours of growth in 20 nM [^{3}H]5-methyltetrahydrofolate, the folate-deficient cells have repleted their folate content to a value equivalent to cells grown in folate-complete medium. The intracellular folate content was approximately 3 μM which was 150 times greater than the extracellular concentration of folate. The cells grown in folate-complete medium, washed with folate-deficient medium, and then incubated in the presence of the radiolabeled 5-methyltetrahydrofolate bound only small amounts of and accumulated less than 10% of the radioactive folate bound by folate-deficient cells. Similarly, folate-deficient cells, after incubation for 3 hours at 37°C with 5-methyltetrahydrofolate, did not bind significant quantities of [^{3}H]5-methyltetrahydrofolate ($<$10% of folate-deficient cells).

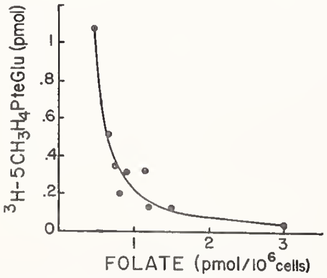


FIGURE 2.—Effect of cell folate content on binding of 5-methyltetrahydrofolate. MA104 cells were initially plated and grown as in figure 1. Every 24 hr for 3 days, the cell folate content and binding of [^{3}H]5-methyltetrahydrofolate at 4°C was determined. Total cell folate was measured as described elsewhere (*4*). Reprinted with permission from Proc Natl Acad Sci USA 83:5983–5987, 1986.

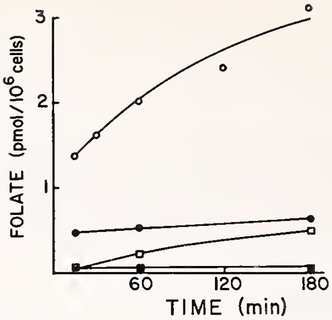


FIGURE 3.—Folate binding and uptake by folate-deficient and folate-replete cells. MA104 cells were grown in folate-deficient (*circles*) or folate-complete medium (*squares*) for 72 hr and then binding at 4°C (*closed symbols*) and accumulation at 37°C (*open symbols*) was determined. Controls, as described in figure 1, were done for all conditions.

Since proteins with a high affinity and specificity for folate have been found in milk and serum as well as tissue in vivo and more specifically, cell membranes (*5–11*), we studied the effects on the binding of [^{3}H]5-methyltetrahydrofolate of a purified FBP from porcine plasma and of a rabbit antiserum against this protein. When [^{3}H]5-methyltetrahydrofolate was incubated for 5 minutes at room temperature with the FBP, subsequent cell binding of the folate, measured after a 1-hour incubation at 4°C, was decreased by $>$90%. Rabbit anti-FBP, incubated with cells for 1 hour at 4°C, decreased [^{3}H]5-methyltetrahydrofolate binding by up to 80% in a dose-dependent fashion (100 to 600 μl antiserum), whereas preimmune serum had no effect (*4*).

We conclude from these initial observations that prior studies of methotrexate and folate transport in vitro failed to identify a highly specific, high-affinity receptor for folate because of the experimental conditions used. Specifically, the cells were grown in extraordinarily high concentrations of folic acid which were, presumably, saturating or causing a "biologic down-regulation" of the synthesis of the membrane receptor for folate. Cells grown in low (physiologic) concentrations of folate have a saturable, high-affinity membrane receptor for folate and analogs such as methotrexate. Based upon the results shown here, the receptor-mediated process is capable of repleting cells to a normal intracellular folate content, thus helping the cell to maintain a concentration gradient of 150 to 200 times over the folate content in plasma.

Since folate is an essential growth requirement, especially for the synthesis of purines and thymidine, necessary components for DNA synthesis, if the receptor is related to cellular proliferative capacity, or regulated by critical events in the cell cycle, it may be that specific delivery of antifols, such as methotrexate, to a distinct population of cells can be more selectively achieved using lower concentrations of methotrexate rather than the higher doses currently in use. In addition, just as there are already tightly binding inhibitors of nucleoside receptors, this folate receptor could be another potential target for anticancer therapy.

REFERENCES

(*1*) SIROTNAK FM: Obligate genetic expression in tumor cells of a fetal-membrane property mediating "folate" transport:

Biological significance and implications for improved therapy of human cancer. Cancer Res 45:3992–4000, 1985.

(*2*) HENDERSON GB, ZEVELY EM: Characterization of the multiple transport routes for methotrexate in L1210 cells using phthalate as a model anion substrate. J Membr Biol 85:263–268, 1985.

(*3*) KAMEN BA, CASTON JD: Direct radiochemical assay for serum folate: Competition between [^{3}H]folic acid and 5 methyltetrahydrofolic acid for a folate binder. J Lab Clin Med 83:164–172, 1974.

(*4*) KAMEN BA, CAPDEVILA A: Receptor-mediated folate accumulation is regulated by the cellular folate content. Proc Natl Acad Sci USA 83:5983–5987, 1986.

(*5*) ANTONY A, UTLEY CS, MARCELL PD, et al: Isolation, characterization, and comparison of the solubilized particulate and soluble folate-binding proteins from human milk. J Biol Chem 257:10081–10089, 1982.

(*6*) KAMEN BA, CASTON JD: Purification of folate-binding factor in normal human umbilical cord serum. Proc Natl Acad Sci USA 72:4261–4264, 1975.

(*7*) SELHUB J, FRANKLIN WA: The folate-binding protein of rat kidney. J Biol Chem 259:6601–6606, 1984.

(*8*) KAMEN BA, CASTON JD: Properties of a folate-binding protein (FBP) isolated from porcine kidney. Biochem Pharmacol 35:2323–2329, 1986.

(*9*) MCHUGH M, CHENG YC: Demonstration of a high-affinity folate binder in human cell membranes and its characterization in cultured human KB cells. J Biol Chem 254: 11312–11318, 1979.

(*10*) ANTONY A, UTLEY C, VAN HORNE KC, et al: Isolation and characterization of a folate receptor from human placenta. J Biol Chem 256:9684–9692, 1981.

(*11*) KAMEN BA, HOLCENBERG JS, TURO K, et al: Methotrexate and folate content of erythrocytes in patients receiving oral vs. intramuscular therapy with methotrexate. J Pediatr 104:131–133, 1984.

Pharmacokinetics of Leucovorin (D,L-5-Formyltetrahydrofolate) After Intravenous Injection and Constant Intravenous Infusion[1]

J. A. Straw,[2,*] E. M. Newman,[3] and J. H. Doroshow[4]

ABSTRACT—The pharmacokinetics of the active and inactive diastereoisomers of leucovorin and its active metabolite, 5-methyltetrahydrofolate (5-CH$_3$-THF) were studied after iv injection of leucovorin in normal human subjects at a dose of 28 mg/m^2, and in patients given 500 mg/m^2 daily by constant iv infusion for a 5.5 day period. In both studies the plasma half-life (t½) of the active isomer, L-formyltetrahydrofolate (CHO-THF), was only 32 to 35 minutes, whereas the inactive isomer, D-CHO-THF had a plasma t½ of 352 to 485 minutes. During constant infusion, the plasma levels reached plateaus of 2.33 and 37.5 μM for L-CHO-THF and D-CHO-THF, respectively. The inactive isomer was cleared from plasma only by urinary excretion of the unchanged drug. The active isomer was also excreted unchanged in the urine but in addition was extensively metabolized to the active metabolite L-5-CH$_3$-THF. The active metabolite achieved a plasma level of 4.85 μM during constant infusion and appeared to have a longer t½ after constant infusion than was observed after iv injection. Furthermore a larger apparent volume of distribution (Vd) of 5-CH$_3$-THF was obtained in the constant infusion study. These findings suggest that constant iv infusion of large doses of leucovorin can considerably expand the intracellular pools of active folate. The consequence of the extensive accumulation of the inactive isomer, D-CHO-THF, is not known. However, the small Vd of D-CHO-THF suggests that it does not extensively accumulate in tissues.—NCI Monogr 5:41-45, 1987.

The combination of FUra and leucovorin is currently undergoing clinical trials in a number of centers (*1*). The biochemical rationale for this combination presumes that administration of large doses of leucovorin will increase the endogenous pools of active folates and enhance the formation and/or stability of the thymidylate synthetase-5-fluorodeoxyuridine monophosphate-methylenetetrahydrofolate complex. A knowledge of the pharmacokinetic behavior of leucovorin and its active metabolite is essential if one is to select doses of leucovorin which will produce adequate plasma levels of active folates.

Leucovorin calcium is the soluble calcium salt of D,L-CHO-THF. The commercially available products are prepared by chemical rather than enzymatic reduction and therefore consist of equal amounts of the diastereoisomers. It is generally accepted that only the natural isomer is active as a cofactor (*1,2*). In this paper we compare observations on the pharmacokinetics of the diastereoisomers studied after iv injection in normal human subjects to some preliminary findings in which high doses of leucovorin were given by constant infusion over a 5.5-day period.

MATERIALS AND METHODS

Subjects

Twelve normal healthy subjects participated in the studies involving iv injection of leucovorin. Two patients with colorectal and one with hepatocellular carcinoma enrolled in phase II clinical trials received leucovorin (500 mg/m^2/day) for 5.5 days. These patients also received FUra (370 mg/m^2) by iv bolus daily for 5 days beginning 24 hours after leucovorin infusion was started.

Protocol

For iv administration, leucovorin (Leucovorin Calcium; Lederle) was dissolved in 10 ml of 5% dextrose and infused at a rate of 2.2 ml/minute (infusion time was 4.5 min). Blood samples were taken prior to drug administration, and at 30 minutes, 1, 2, 4, 6, 8, 10 and 24 hours after iv infusion. Urine was collected for 4 to 12 hours after iv infusion.

For the constant infusion studies, the dose of leucovorin for each 12 hours (250 mg/m^2) was dissolved in 1 liter of 5% dextrose and infused into a peripheral vein at 85 ml/hour using an IMED infusion pump, model 960 (IMED Corp, San Diego, CA). The solution was protected from light during the infusion. Blood samples were taken from the opposite arm prior to the infusion. After the infusion began, samples were taken at 10, 20 and 30 minutes, 2, 4, 6 and 24 hours, at 24-hour intervals during the infusion, and at the same time points for the first 6 hours after the end of the infusion. Urine was collected for two 24-hour periods during steady state.

Chemicals

Culture media were obtained from Difco Laboratories, Detroit, MI. D,L-CHO-THF and D,L-5-CH$_3$-THF, used for

---

ABBREVIATIONS: AUC = area under curve; C = concentration at the last data point; C$_0$ = zero time concentration; CHO-THF = formyltetrahydrofolate; 5-CH$_3$-THF = 5-methyltetrahydrofolate; Clp = plasma clearance; Clr = urinary clearance; HPLC = high-pressure liquid chromatography; K = elimination rate constant; t½ = half-life; TBA = tetrabutyl ammonium hydroxide; V$_c$ = volume of central compartment; Vd = apparent volume of distribution.

---

[1] Supported by a grant from American Cyanamid Co., Lederle Laboratories, Pearl River, NY, and by NIH BRSG S07RR-05471 to the City of Hope National Medical Center.

[2] Department of Pharmacology, The George Washington University, Washington, DC.

[3] Division of Pediatrics, and [4] Department of Medical Oncology and Therapeutics Research, City of Hope Medical Center, Duarte, CA.

[*] *Reprint requests to:* J. A. Straw, Ph.D., Department of Pharmacology, The George Washington University, 2300 Eye Street, NW, Washington, DC 20037.

high-pressure liquid chromatography (HPLC) standards, were purchased from Sigma Laboratories, St. Louis, MO. L-CHO-THF, used as the standard for the microbiological assays, was generously provided by R. Moran, Childrens Hospital of Los Angeles. Other chemicals and solvents were of standard laboratory grade except for the solvents used for HPLC which were HPLC grade.

Analytical Procedures

For the iv injection studies, HPLC analysis of plasma and urine samples was performed as described previously (*3*). Briefly, the method involved gradient separation using tetrabutyl ammonium hydroxide (TBA) in 30 to 60% methanol on a C-18 5-micron column and UV detection at 300 nm of 5-CH_3-THF and D,L-CHO-THF.

The biologically active form of leucovorin (L-CHO-THF) was measured in plasma and urine samples by the disc assay method of Mehta and Hutchison (*4*) and the concentration of D-CHO-THF was calculated as the difference between the concentration of D,L-CHO-THF as measured by HPLC and L-CHO-THF measured by microbiological assay.

For the constant infusion studies, plasma and urine samples were analyzed for biologically active folates using a new semi-automated method (*5*). L-CHO-THF was measured directly, using *Pediococcus cerevisiae,* and 5-CH_3-THF was measured by *Lactobacillus casei* bioassay of appropriate HPLC fractions obtained by a modification of the method described above. In addition, the urine samples were subjected to HPLC analysis using a step-gradient system in which 10 ml of 1.6 m*M* TBA in 10% methanol (pH 2.0) was used to elute ascorbic acid and other interfering substances followed by 1.6 m*M* TBA in 30% methanol (pH 4.1) to elute and separate the folates. The system used a 10 micron C-18 column and detection at 294 nm and gave superior separation of folic acid from the reduced folates than the method previously described (*3*). The concentrations of D-CHO-THF in plasma and urine samples were calculated as described above.

Data Analysis

IV injection studies.—The plasma concentration versus time data for the elimination phases of L-CHO-THF, D-CHO-THF, and 5-CH_3-THF were fit to the equation $C=C_0e^{-Kt}$ by the method of least squares to calculate the elimination rate constants (K) and t½ for each subject, where C is the concentration at the last data point, and C_0 is the zero time concentration.

The area under curve (AUC) was calculated using the observed values at each time point and the trapezoid method. The C_0 of L-CHO-THF and D-CHO-THF were estimated by extrapolation and the C_0 of 5-CH_3-THF was taken as zero. The area from the final data point to infinity was estimated as C/K.

Urinary clearance (Clr) was calculated using:

$$\mathrm{Clr}=\text{amount in urine (0 to time t)}/\text{AUC (0 to time t)}.$$

Plasma clearance (Clp) was calculated using:

$$\mathrm{Clp}=\text{dose}/\text{AUC}.$$

The Vd was calculated using:

$$\mathrm{Vd}=\mathrm{Clp}/\mathrm{K}.$$

To calculate the Clp and Vd for 5-CH_3-THF, it was necessary to make an assumption concerning the dose of 5-CH_3-THF (i.e., the amount of L-CHO-THF metabolized to 5-CH_3-THF). The upper limit (75%) is obtained if one assumes that all L-CHO-THF not excreted in the urine is metabolized to 5-CH_3-THF. The lower limit (35%) is obtained if one assumes that the fate of all of the 5-CH_3-THF metabolized from L-CHO-THF was excretion in the urine. The true value lies somewhere within these extremes; the mean of the two values (55%) was used.

Constant infusion studies.—The concentration versus time data from start of the infusion to 6 hours postinfusion for D-CHO-THF and 2 hours postinfusion for L-CHO-THF was fit to the following equation for constant infusion and first order decay for a one compartment model using PCNONLIN (Statistical Consultants, Lexington, KY):

$$\mathrm{C(T)=(D/T_I)/Vd/K(EXP(-KT_{STAR})-EXP(-KT))},$$

where D = total dose, T_I = time of infusion, and $T_{STAR}=T-T_I$ for $T>T_I$ and $T_{STAR}=0$ for $T<T_I$.

The dose was taken as 1/2 the infusion rate of D,L-CHO-THF and the parameters Vd and K were varied to achieve the best fit.

The concentration versus time data for 5-CH_3-THF from start of the infusion to 6 hours postinfusion was fit to a two-compartment model described by the following system of equations:

$$d(5\text{-}CH_3\text{-}THF)_1/dt=\mathrm{KFC}-(\mathrm{K10}+\mathrm{K12})(5CH_3\text{-}THF)_1+\mathrm{K21}(5\text{-}CH_3\text{-}THF)_2$$
$$d(5\text{-}CH_3\text{-}THF)_2/dt=\mathrm{K12}(5CH_3\text{-}THF)_1-\mathrm{K21}(5\text{-}CH_3\text{-}THF)_2$$

where: $(5\text{-}CH_3\text{-}THF)_1$ = the concentration in compartment one, $(5\text{-}CH_3\text{-}THF)_2$ = the concentration in compartment two, and the input function (KFC) is the elimination rate constant for L-CHO-THF times the fraction of L-CHO-THF assumed to be metabolized times the plasma concentration of L-CHO-THF as described by the equation above.

The parameters, varied to obtain the best fit, were the micro rate constants K10, K12, and K21 which were used to calculate elimination rate constant β. The volume of the central compartment (Vc) was assumed to be the same as the Vd of L-CHO-THF. This assumption implies that 5-CH_3-THF is produced in and removed from a pharmacokinetic compartment identical to that defined by the pharmacokinetic behavior of L-CHO-THF. Clp was calculated as the product of K10 and Vc. Vd(beta) was calculated as Clp/β.

Clr was calculated for 24-hour excretion during steady state using the equation: Clr = amount excreted/plasma concentration.

RESULTS

iv Injection

As shown in figure 1, the unnatural isomer (D-CHO-THF) had a relatively long t½, and plasma concentrations greatly exceeded those of L-CHO-THF at all time points. The rapid disappearance of L-CHO-THF was accompanied by the appearance of 5-CH_3-THF, which decayed with a t½ much longer than the parent compound.

Table 1 shows the calculated pharmacokinetic parameters for the three compounds. Comparison of the Clp and Clr of D-CHO-THF shows that the unnatural isomer did

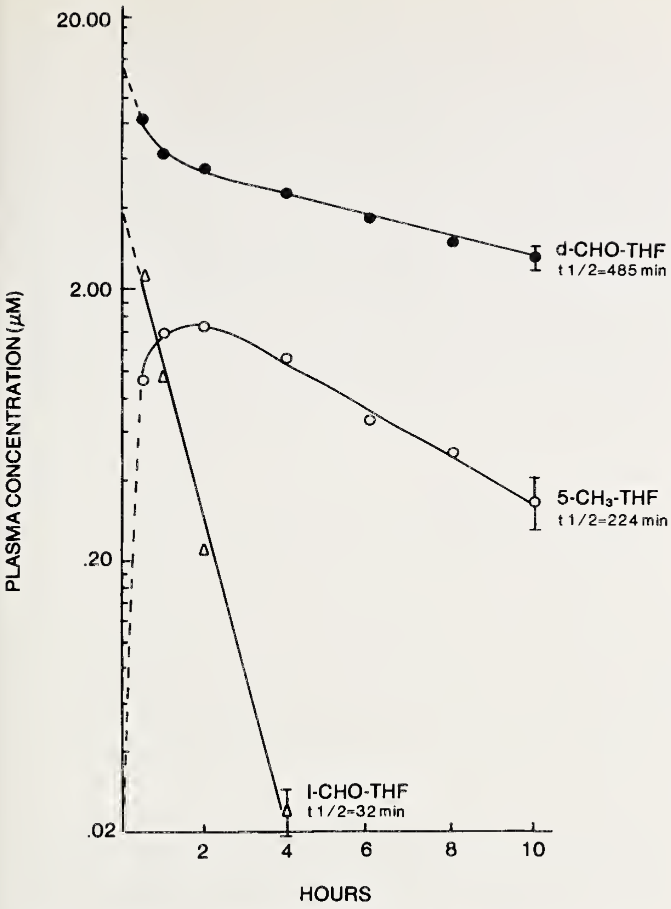


FIGURE 1.—Plasma concentrations of D-CHO-THF (●), L-CHO-THF (△), and $5\text{-}CH_3$-THF (○) after iv injection of 28 mg/m^2 leucovorin. Points represent mean values obtained in 12 subjects. Bars = ± SE.

not undergo metabolism because urinary excretion of the unchanged drug accounted for total body elimination of this compound.

Constant iv Infusion

In figure 2 the lines depict the simulated fit to the data points. A good fit to the L-CHO-THF and D-CHO-THF data points was achieved using a 1-compartment model. A 2-compartment model was required to fit the $5\text{-}CH_3$-THF data. The postinfusion decay curves for D-CHO-THF and L-CHO-THF are similar to those obtained after iv injection, but $5\text{-}CH_3$-THF had a longer t½ after infusion.

The last two data points on the L-CHO-THF plasma decay curve suggest that a slow terminal phase was present. However, this might not be true because the bioassay for L-CHO-THF could not be shown to be specific for L-CHO-THF in the presence of 500- to 1000-fold excess D-CHO-THF (*6*). Furthermore, attempts to fit the data to a two compartment model using the last two data points resulted in a poor fit to data points measured when plasma levels of D-CHO-THF did not interfere with the bioassay.

Table 2 shows the pharmacokinetic parameters calculated for the infusion study. When compared to the iv injection results, no significant differences were observed for the parameters calculated for D-CHO-THF. Clp and Clr were not significantly different (88 to 100% of the dose was excreted in the urine) in the infusion study.

Examination of the pharmacokinetic parameters for L-CHO-THF shows that the Clp of this compound was significantly lower in the constant infusion study than in the iv injection study, whereas the Clr did not differ in the two studies. Thus, nonrenal clearance of L-CHO-THF during constant infusion was less than that observed after iv injection. It is possible that metabolism of L-CHO-THF was saturated in the infusion study but additional studies using lower and higher infusion rates would be required to verify this possibility.

The pharmacokinetics of $5\text{-}CH_3$-THF determined during the iv infusion study differed in several ways from those obtained after iv injection. These differences can be explained in part by the failure to observe the biphasic plasma decay and 2-compartment behavior in the iv injection study, because plasma levels of $5\text{-}CH_3$-THF approached the limits of detection before the slower terminal phase clearly developed. This would explain the observation that the plasma t½ obtained in the infusion study was almost double that seen in the iv injection study. Calculation of both Clp and Vd involved the assumption that 55% of L-CHO-THF was metabolized to $5\text{-}CH_3$-THF. Because we cannot be assured that the percent of L-CHO-THF metabolized to $5\text{-}CH_3$-THF was the same under the two different experimental conditions, it is inappropriate to attempt to compare these derived parameters in the two different experiments. Moreover, Clr, which was calculated directly and required no assumptions, was about two fold greater in the constant-infusion study which suggests that metabolic disposition of $5\text{-}CH_3$-THF may be different under the two experimental conditions.

For the constant-infusion study, the Vd of $5\text{-}CH_3$-THF was also calculated from Clr. This gave an estimate of the Vd that would be associated with the amount of $5\text{-}CH_3$-THF recovered during a 24-hour period at steady-state plasma levels. This calculation provided the minimum estimate of the Vd of $5\text{-}CH_3$-THF. Nevertheless this value

TABLE 1.—Pharmacokinetic parameters obtained in 12 normal subjects following iv injection of leucovorin 28 mg/m^2

	Plasma concentration, μM[a]	t1/2, min	Clp, ml/min	Clr, ml/min	Clnr,[b] ml/min	Vd, liters
D-CHO-THF	7.85[c] ± .73	485 ± 35	12.7 ± 1.0	12.7 ± 1.4		7.9 ± 0.50
L-CHO-THF	2.2 ± .021	32.0 ± 1.8	390 ± 14	86.0 ± 6.4	294 ± 27.8	16.4 ± 1.1
$5\text{-}CH_3$-THF	1.28 ± .14	224 ± 28	54.7[d] ± 6.4	32.2 ± 2.9	22.5 ± 4.55	16.7[d] ± 2.2

[a] Plasma concentration 30 min postinjection for L-CHO-THF, D-CHO-THF, and 2 hours postinjection for $5\text{-}CH_3$-THF.

[b] Clnr = nonrenal clearance.

[c] Mean ± SE.

[d] This value was calculated assuming that 55% of L-CHO-THF was metabolized to $5\text{-}CH_3$-THF.

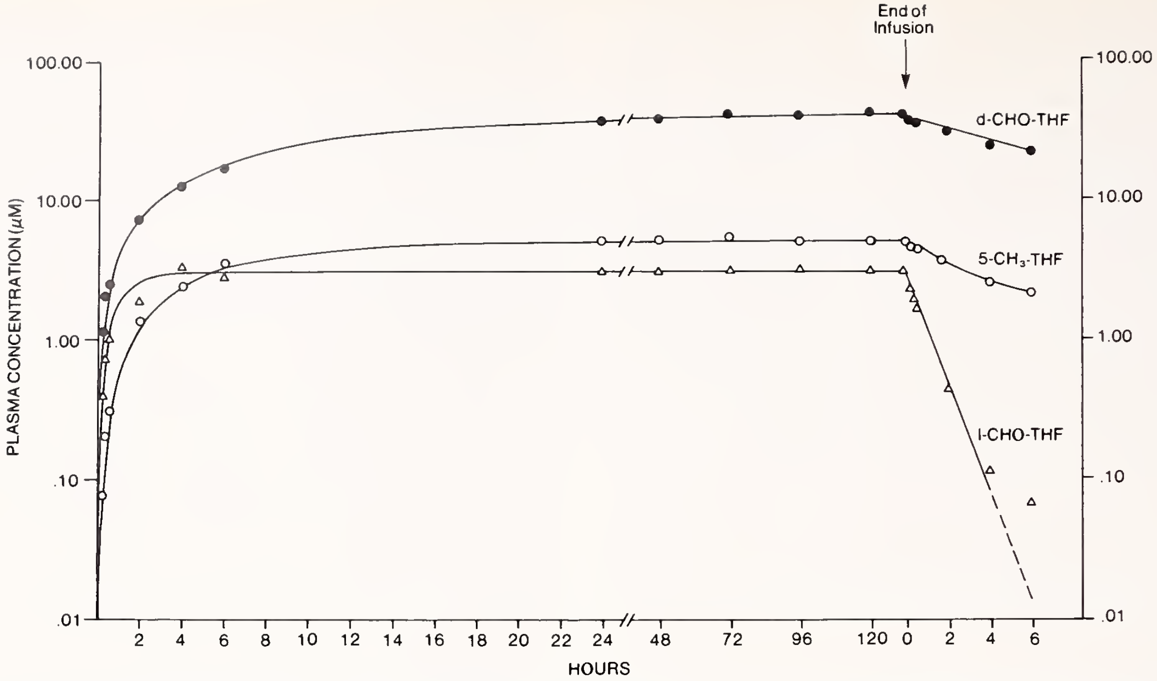


FIGURE 2.—Plasma concentrations of D-CHO-THF (●), L-CHO-THF (△), and 5-CH_3-THF (○) during and after constant infusion of leucovorin 500 mg/m^2 for 5.5 days. Points represent mean values obtained in 3 subjects. Lines were generated by computer fit as described in Methods.

(38.2 liters) was almost 3 times the Vd calculated for L-CHO-THF ($P < .01$) and indicates that, under conditions of constant iv infusion of leucovorin, the metabolite accumulated in excess of the parent compound.

DISCUSSION

A limited number of patients have been studied during constant infusion and additional patients should be studied. Nonetheless, the preliminary data suggest that the following comparisons between the rapid iv injection and the continuous iv infusion studies will pertain.

The two studies are entirely consistent with respect to findings for the unnatural isomer (D-CHO-THF). The following conclusions concerning this isomer seem to be justified: a) During constant iv infusion of leucovorin, D-CHO-THF achieves steady-state plasma levels greatly in excess of the active isomer or of its metabolite (5-CH_3-THF). b) It does not undergo metabolic degradation and is excreted unchanged in the urine.

Care must be used in interpretation of estimates of Vd. The Vd estimates for both D- and L-CHO-THF were made assuming a 1-compartment model because the data did not lend itself to description of a more elaborate model. This does not mean that these compounds distributed in only 1 compartment, but does suggest that equilibration between a central and peripheral compartment was too rapid to allow 2-compartment analysis with the data collected.

Because the data for 5-CH_3-THF required a 2-compartment model to achieve a reasonable fit to the data, it was possible to estimate the intercompartmental rate constants from the constant-infusion study. However, interpretation of the data is severely hampered by our inability to assign a value to the dose of 5-CH_3-THF. We have attempted to circumvent this by using assumptions which set the upper and lower limits for the amount of L-CHO-THF converted to 5-CH_3-THF. It is likely that the actual amount of L-CHO-THF metabolized to 5-CH_3-THF lies somewhere between these extremes and the amount may differ depending on the dose of L-CHO-THF. Nevertheless, the data indicate that during constant infusion of L-CHO-THF, 5-CH_3-THF is extensively accumulated in the body. Except in special circumstances, pharmacokinetically derived compartments do not define anatomical spaces. Therefore,

TABLE 2.—Pharmacokinetic parameters obtained in 3 subjects receiving leucovorin by constant iv infusion at a dose of 500 mg/m^2/24 hours for 5.5 days

	C_{ss},[a] μM	t1/2, min	Clp, ml/min	Clr, ml/min	Clnr, ml/min	Vd, liters
D-CHO-THF	37.5[b] ± 8.4	352 ± 84	18.0 ± 3.7	16.3 ± 3.1	1.4 ± 1.0	8.5 ± 1.1
L-CHO-THF	2.33 ± 0.5	35.3 ± 7.8	280[c] ± 41.5	78.8 ± 14.5	204 ± 30.8	13.6 ± 2.5
5-CH_3-THF	4.85 ± 1.3	412 ± 3.4	78.6[d] ± 16.2	62.8[c] ± 13.0	15.8 ± 8.1	46.6[d] (38.2)[e] ± 17.7 (±8.2)

[a] C_{ss} = steady-state plasma concentration.

[b] Mean ± SE.

[c] Significiantly different from value obtained after a single iv injection, $P < .01$.

[d] This value was calculated assuming that 55% of L-CHO-THF was metabolized to 5-CH_3-THF.

[e] This value for Vd was calculated assuming that all 5-CH_3-THF produced from L-CHO-THF was excreted unchanged in the urine.

it is likely that the relatively large Vd for 5-CH_3-THF, compared to that of 5-CHO-THF, represents concentration of this folate in tissues, possibly in the form of polyglutamates.

The unnatural isomer (D-CHO-THF) achieved the highest plasma concentration at steady state but had a relatively small Vd. It would appear that the accumulation of D-CHO-THF is the result of decreased renal excretion rather than accumulation in intracellular sites. The consequences of the relatively high plasma levels of this isomer are unknown. Potential sites of interaction with the natural isomers include reabsorption in the renal tubule, uptake into cells, and formation of polyglutamates. Studies using the active isomer alone are needed to answer these questions regarding D-CHO-THF.

REFERENCES

(*1*) BRUCKNER HW: Summary: Status of 5-FU–leucovorin combination. *In* Advances in Cancer Chemotherapy: The Current Status of 5-Fluorouracil-Leucovorin in Calcium Combination (Bruckner HW, Rustum YM, eds). New York: Park Row, 1984, pp 88–89.

(*2*) BLAKLEY RL: The Biochemistry of Folic Acid and Related Pteridines. New York: American Elsevier, 1969, p 82.

(*3*) STRAW JA, SZAPARY D, WYNN WT: Pharmacokinetics of the diastereoisomers of leucovorin after intravenous and oral administration to normal subjects. Cancer Res 44:3114–3119, 1984.

(*4*) MEHTA B, GISOLFI AL, HUTCHISON DJ, et al: Serum distribution of CF and 5-methyltetrahydrofolate following oral and im administration of calcium leucovorin in normal adults. Cancer Treat Rep 62:345–350, 1978.

(*5*) NEWMAN EM, TSAI JF: Microbiological analysis of 5-formyltetrahydrofolic acid and other folates using an automatic 96-well plate reader. Anal Biochem 154:509–515, 1986.

(*6*) SATO JK, NEWMAN EM, MORAN RG: Preparation of (6R)-tetrahydrofolic acid and (6R)5-formyltetrahydrofolic acid of high stereochemical purity. Anal Biochem 154:516–524, 1986.

Absorption Kinetics of Orally Administered Leucovorin Calcium[1]

Brian W. McGuire,[2,*] Luisa L. Sia,[3] John D. Haynes,[3] James C. Kisicki,[4] Manuel L. Gutierrez,[5] and E. L. Robert Stokstad[6,7]

ABSTRACT—Oral dose proportionality and pharmacokinetics of leucovorin [(d,l)-5-formyltetrahydrofolate (5-formyl-THF)] were studied in 30 healthy male subjects. In a randomized crossover design, 24 fasted subjects were given 4 of a series of 5 single test doses between 20 and 100 mg, at 1-week intervals, of 5-formyl-THF as an oral solution of leucovorin calcium. Six separate subjects received 200 mg iv and po in a 2-way crossover. Blood and urine samples were collected over 24 hours for differential microbiological folate assays using *Lactobacillus casei* and *Streptococcus faecalis*.

Using *L casei* activity to measure total serum folates, the area under the concentration-time curve from 0 to infinite time (AUC[0 – ∞]) was calculated. Relative bioavailabilities were 78%, 62%, 49%, and 42% for the 40-, 60-, 80-, and 100-mg doses, respectively. Both the AUC and peak concentration (CPEAK) of total folates (consisting predominantly of the major metabolite, 5-methyltetrahydrofolate (5-methyl-THF)), displayed significant deviation from linearity consistent with a saturation of folate absorption. Absolute bioavailability of the 200-mg oral dose of leucovorin based on AUC was 31% compared with that of the iv dose (6,848 vs. 22,298 ng·hr/ml, respectively). Total clearance, terminal half-life, and apparent volume of distribution of total folate at the 200-mg dose were not significantly different between the two routes of administration. Eighty-three percent of the biologically active iv dose was recovered in the urine within 24 hours, 31% as 5-methyl-THF. Twenty percent of the same oral dose was excreted in 24 hours, 16% as 5-methyl-THF. In contrast to the nondose-proportionality observed in total serum folates, AUC of the small component of *S faecalis* activity, which appeared earlier than 5-methyl-THF, displayed linear kinetics, suggestive of a distinct mechanism of uptake. As dose increased, *S faecalis* activity increased in relative proportion to *L casei,* indicating that saturation of the enzymatic bioconversion to 5-methyl-THF may also be occurring. In light of the demonstrated nondose-proportionality of total folates with oral leucovorin in this dose range, consideration should be given to parenteral administration in regimens employing higher doses. Oral administration should be at a level consistent with the capacity for efficient folate uptake.—NCI Monogr 5:47–56, 1987.

Leucovorin, or 5-formyl-THF, also known as folinic acid or citrovorum factor, is one of several biologically active forms of the reduced folic acid vitamin. The various derivatives of tetrahydrofolate (THF) play an integral role in single-carbon transfers among a variety of biological compounds. The pharmaceutical preparation leucovorin calcium, a racemic mixture, is indicated for certain folic acid deficiencies but primarily for the counteraction of toxicities encountered following administration of folate antagonists (e.g., methotrexate). The net impact of leucovorin administration, whether oral or parenteral, is the expansion of the total reduced folate pool and subsequent utilization of the folate coenzymes in a broad spectrum of biochemical processes including de novo nucleotide synthesis, amino acid metabolism, and other reactions involving single-carbon donors and acceptors.

Not completely elucidated are certain facets of gastrointestinal folate transport and mucosal metabolism. Hepner et al. (*1*), using an in vivo double-lumen perfusion technique, demonstrated in normal subjects that pteroylglutamic acid (unreduced folic acid) was principally absorbed in the proximal small intestine or upper jejunum; there was little uptake in the distal jejunum, and none in the ileum. Transport could occur against a concentration gradient. As the concentration of the folate solution was increased (between 10 and 10,000 ng/ml), the percent absorbed decreased. A nonlinear active transport process was suggested by these data, although others have invoked a mechanism of facilitated diffusion for folate absorption (*2,3*).

Further evidence of a nonlinear or saturable uptake process was provided by Straw et al. (*4*), who investigated 3 oral leucovorin dose levels (multiple 25-mg doses and single 50- and 100-mg doses) in a small number of subjects. Apparent availability of the 25-mg dose was estimated to be near 100%; with increasing dose, however, availability declined. They also reported a stereoselectivity of absorption for the biologically active (l)-isomer of leucovorin over the inactive (d)-isomer. These observations pointed to a specific saturable carrier-mediated absorption process for folates. Their findings also indicated that no conversion of

---

ABBREVIATIONS: ANOVA = analysis of variance; AUC[0–x] = area under concentration-time curve from 0 to x hours; Cl = total clearance; Cl_R = renal clearance; CPEAK = peak concentration; CV = coefficient of variation; 5-FdUMP = 5-fluorodeoxyuridylate; 5-formyl-THF = 5-formyltetrahydrofolate; K_{EL} = elimination rate constant; 5-methyl-THF = 5-methyltetrahydrofolate; THALF = terminal half-life; THF = tetrahydrofolate; TPEAK = time to peak concentration; VD_β = volume of distribution.

---

[1] Supported by the Department of Clinical Development, Lederle Laboratories, Division of American Cyanamid Co.

[2] Clinical Research Department, Alpha Therapeutic Corporation, Los Angeles, CA.

[3] Department of Statistical Design and Analysis, Medical Research Division of American Cyanamid, Pearl River, NY.

[4] Harris Laboratories Inc., Lincoln, NE.

[5] Department of Clinical Development, Lederle Laboratories, Division of American Cyanamid, Pearl River, NY.

[6] Department of Nutritional Sciences, University of California, Berkeley, CA.

[7] We thank D. Caesar, J. Eatherton, and S. Meachum for technical assistance, and C. McGuire for aid in manuscript preparation.

[*] *Reprint requests to:* Department of Clinical Development, Lederle Laboratories, Division of American Cyanamid Co., Pearl River, NY 10965.

(d)-5-formyl-THF took place but that this inactive isomer was cleared solely by means of a slow renal excretion.

In clinical practice, leucovorin is given following methotrexate in the range of 10 to 25 mg in a series of doses every several hours. Regimens employing much higher doses in conjunction with the cytotoxic agent 5-fluorouracil are currently under investigation.

Further exploration of the pharmacokinetics of leucovorin is warranted, not only in the context of methotrexate rescue but also in the trend toward high doses of leucovorin which are given with 5-fluorouracil. The present investigation was designed to more completely characterize by means of a series of incremental oral doses the kinetics of folate absorption and to define the dose proportionality or absorptive capacity for leucovorin in humans. Pharmacokinetic characterization of a high iv dose was also undertaken.

MATERIALS AND METHODS

Subjects.—Thirty healthy male volunteers participated in the study: 24 in a 4-way crossover dose-proportionality study and 6 in a high-dose study. The use of human subjects followed Good Clinical Practice guidelines under the auspices of the Harris Laboratories Institutional Review Board. All subjects granted written informed consent prior to participation.

Subjects were screened for inclusion and deemed eligible based on medical history, physical examination, and laboratory work including blood chemistries, hematology, and urinalysis. Mean age of the subjects was 26 years (range: 19 to 45); body weight averaged 77.5 kg (range: 61.7 to 92).

Test drug and procedures.—Leucovorin Calcium Cryodesiccated Powder is a product of Lederle Laboratories. This preparation consists of equal amounts of the (d,l)-diastereoisomers of 5-formyl-THF, of which the (l)-isomer is biologically active. For oral administration, leucovorin calcium was reconstituted with aromatic elixir USP to a final concentration of 1 mg leucovorin free acid per ml solution. For parenteral injection, bacteriostatic water USP was used as diluent for a final concentration of 10 mg/ml. Each preparation was used immediately.

The subjects were randomized to receive 4 of 5 test doses (20, 40, 60, 80, and 100 mg) in 1 of 12 balanced series of sequences. All subjects received the lowest dose (20 mg) and the second highest dose (80 mg), plus 2 of the 3 remaining doses, creating sample sizes of 24 or 16. The 12 sequence combinations were designed to prevent any confounding effects of dose order, carry-over, etc. The 6 subjects in the high-dose group received iv and oral administration of 200 mg in a randomized sequence.

Subjects fasted from the evening prior to dosing until 4 hours after drug administration. Oral dosing consisted of the assigned volume of leucovorin solution plus additional fluid to match all liquid intake to 250 ml. Those receiving iv administration were given a slow injection (20 ml over 5 min or 4 ml/min) into a peripheral arm vein.

Serial blood samples were drawn at times 0 (predose), 20, 40, 60 minutes, 1.5, 2, 3, 4, 7, 12, and 24 hours. Serum was prepared and stored frozen with 5 mg/ml ascorbate as an antioxidant. Twenty-four-hour urine collection was carried out only for the 6 subjects in the high-dose substudy (predose, 0 to 2, 2 to 4, 4 to 12, and 12 to 24 hr intervals), and ascorbate was added prior to freezing. One week following treatment, subjects were readmitted to the clinic for each successive crossover treatment. Twenty-three original subjects plus 1 dropout replacement completed successfully all 4 crossover periods. All 6 in the high-dose group completed the 2-way crossover.

Microbiological folate assays.—Folates in the biological samples were measured by means of differential microbiological assays utilizing the growth response of the 2 folate-dependent organisms *Lactobacillus casei* (ATCC 7469) and *Streptococcus faecalis* (ATCC 8043), obtained from American Type Culture Collection. Folinic acid (d,l)-5-formyl-THF (Sigma Chemicals) was used as a standard for each microorganism. Complete methods are described elsewhere (*5–8*). The bacterium *L casei* displays a growth response to the natural (l)-stereoisomer of all known pteroylmonoglutamates (unreduced folic acid and all oxidation levels of tetrahydrofolate); *S faecalis* responds to all of these except 5-methyl-THF, a major metabolite of leucovorin (*9*). Since plasma folate is almost exclusively reduced (unreduced folates being of negligible quantity), the *L casei* activity alone measures total tetrahydrofolates, the difference between *L casei* and *S faecalis* corresponds to 5-methyl-THF, and *S faecalis* activity itself measures predominantly formyl-THF. The sensitivity of the microbiological assay for both *L casei* and *S faecalis* extends below 0.5 ng folate/ml serum ($1.0 \times 10^{-9} M$). Reproducibility was measured by determining the standard deviation and coefficient of variation (CV) of 250-ng standard extracts each day of assay. For *L. casei* the CV was 4.8%, for *S. faecalis* 6.9%.

Data analysis.—Pharmacokinetic parameters calculated from the serum folate fractions measured by *L. casei* and *S. faecalis* included peak concentration (CPEAK), time to peak concentration (TPEAK), AUC[0–24] and extrapolated from 0 to infinity (AUC[0 – ∞]), and terminal half-life (THALF). Subjects' baseline (predose) folate values were subtracted from each postdose concentration to adjust for endogenous folate (and for possible carry-over from the previous dose). The trapezoidal rule was used to calculate AUC[0–24]. AUC[0 – ∞] was estimated by the expression:

$$\mathrm{AUC}[0-\infty] = \mathrm{AUC}[0-C_\tau] + \frac{C_\tau}{K_{EL}}$$

in which C_τ is the last available non-zero concentration (which, with 1 exception, was at 24 hr) and K_{EL} the elimination rate constant. Least squares regression was used to estimate K_{EL} using the last 3 non-zero concentrations of the natural log (base e) plot of concentration vs. time (generally 7, 12, and 24 hr). THALF was computed as $\ln 2/K_{EL}$. AUC[0 – ∞] for the iv administration (a 5-min infusion) was adjusted by extrapolating from the first hour concentration determinations (20, 40, 60 min) back to the expected 5-minute CPEAK using linear regression on the natural log of the concentrations at 20, 40, and 60 minutes.

Total clearance (Cl) of folate was calculated from the high-dose iv data as:

$$\mathrm{Cl} = \frac{\mathrm{Dose}}{\mathrm{AUC}[0-\infty]}$$

For the high-dose oral data, the above expression was multiplied by the fraction absorbed (F): $\mathrm{AUC}[0-\infty]_{\mathrm{oral}}/\mathrm{AUC}[0-\infty]_{\mathrm{iv}}$, equal to 0.31.

Volume of distribution (VD_β) was derived from the high-dose IV data by:

$$VD_{\beta} = \frac{Cl}{K_{EL}} = \frac{Dose}{AUC[0-\infty] \times K_{EL}}$$

For the high-dose oral data, the above expression was again multiplied by F of 0.31.

Urinary recovery of folates was determined by multiplying each urine sample concentration by the respective volume from that time interval (0 to 2, 2 to 4, 4 to 12, 12 to 24 hr), summing the values over 24 hours, and expressing as percent of the dose of biologically active ((l)-isomer) folate excreted (i.e., ½ of the actual 200-mg dose, or 100 mg). Renal clearance Cl_R was calculated by dividing folate excreted [0 to 24] by AUC[0 to 24].

In the dose proportionality analysis of the 20 to 100 mg dose range, the AUC and CPEAK values for each subject were dose-adjusted by dividing the value by the respective dose. Resulting means of these and the other pharmacokinetic variables were analyzed statistically by crossover analysis of variance (ANOVA). Subject, period, and dose were included as factors in the linear model. Least-squares means was used to adjust for disparate sample sizes in the different dose groups. In the pairwise comparison of each parameter, *t*-test on the least-squares means was carried out using the ANOVA error term. The statistical power of detecting a 20% difference between AUC[0 – ∞] values of total folates was 98.5%.

RESULTS

Oral Dose Series

Serum concentrations of *L casei* activity (corresponding to total folates) vs. time are shown in figure 1, which superimposes the non-dose-adjusted curves for the 20-, 40-, 60-, 80-, and 100-mg dose levels. Figure 2 shows in histogram form the corresponding AUC[0 – ∞] values calculated (lower bars); the upper bars indicate the theoretical AUC which would result from linear or dose proportional kinetics. As can be seen from both figures, the incremental increases in circulating total folates plateau with increasing dose.

Table 1 lists the absolute AUC[0 – ∞] values, the dose-adjusted means with statistical comparison, and the relative bioavailabilities of the doses based both on the 20-mg dose as reference and on the 200-mg iv dose. The dose-adjusted means are compared statistically in a pairwise fashion with the preceding dose level. The 40-, 60-, and 80-mg means displayed statistically significant decreases from each of their preceding means, illustrative of declining bioavailability with increasing dose. The 100-mg dose-adjusted mean was not significantly different from that of the 80-mg dose, but was significantly less than all other preceding doses.

The close comparability of the 20-mg oral and 200 mg iv doses is noteworthy; these yielded dose-adjusted means of

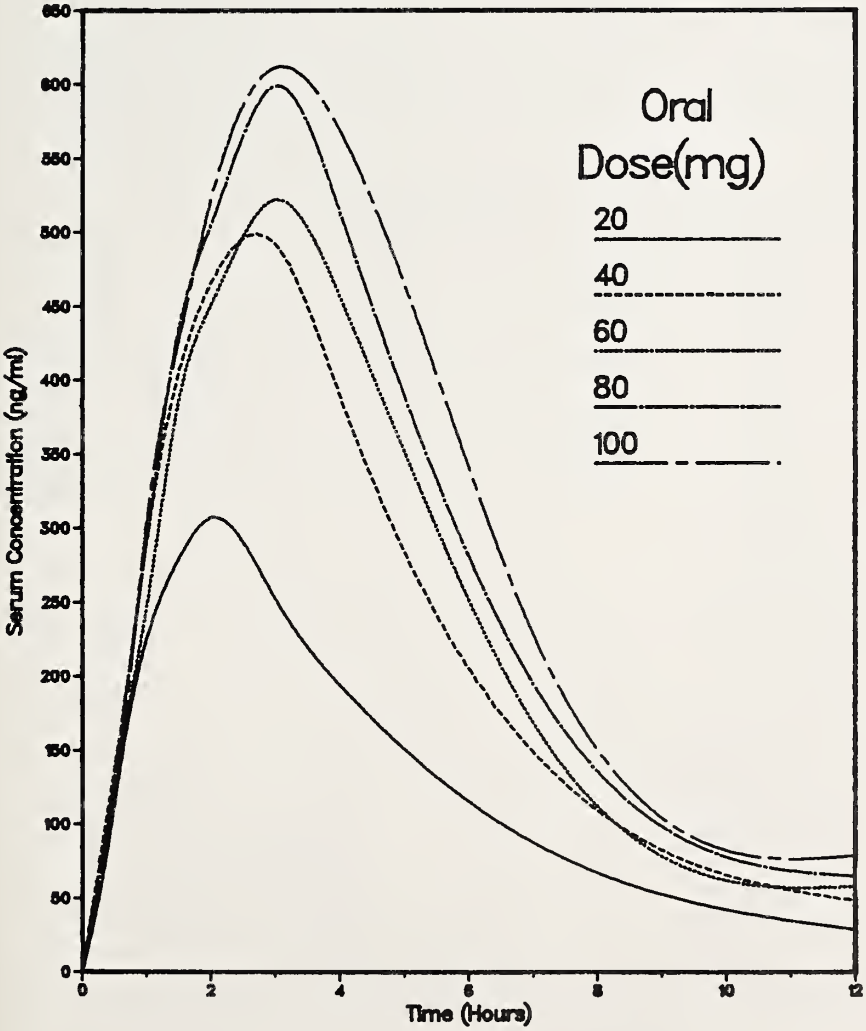


FIGURE 1.—Non-dose-adjusted concentration-time profiles (ng/ml) of total folates (*L casei* activity) after 20-, 40-, 60-, 80-, and 100-mg doses of leucovorin between 0 and 12 hours post-dose (24-hr concentration not shown).

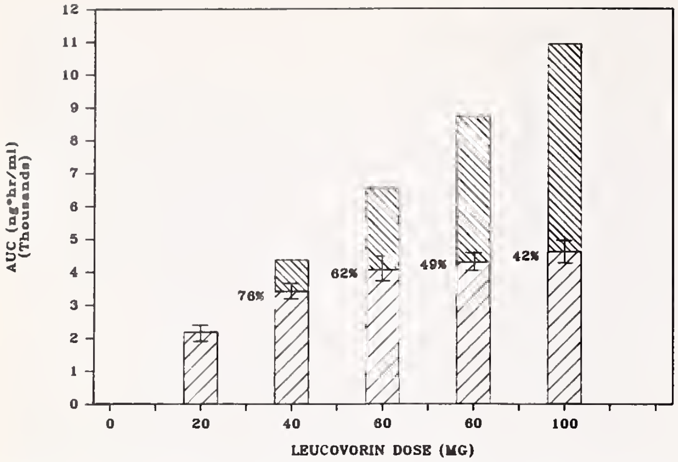


FIGURE 2.—Total folate (*L casei* activity) AUC[0 – ∞] (ng·hr·ml^{-1}) for leucovorin doses between 20 and 100 mg. Lower region is experimental mean; upper region indicates theoretical dose-proportional value. Bar is standard error of the mean. Percent values indicate the bioavailabilities of the experimental doses relative to the dose-adjusted 20-mg total folate AUC[0 – ∞] value.

109.2 and 111.5, respectively. Although performed with different subjects, the result suggests virtually complete (98%) absorption of the 20-mg oral dose. Consequently the relative bioavailabilities of the higher dose levels (table 1) showed the same decline (within 1 to 2%) whether based on the 20-mg oral or 200-mg iv dose as reference.

This drop in relative bioavailability with increasing dose is also indicated in figure 2. Using the 20-mg dose AUC as reference, relative bioavailabilities for the higher doses were, respectively, 78%, 62%, 49%, and 42%. Absolute bioavailability of the 200-mg oral dose was 31% based on the same iv dose.

Peak concentration (CPEAK) of total folates displayed the same nonlinear trend as the AUC data (table 2). Compared with a CPEAK of 318 ng/ml (0.67 μM) obtained from a 20-mg dose, a 5-fold increase in dose produced only a 2-fold increase in CPEAK to 619 ng/ml (1.31 μM). Dose-adjusted means declined significantly in each pairwise comparison.

Differential microbiological assays distinguish by subtraction that component of total folates which is the major metabolite 5-methyl-THF (*L casei* activity minus *S faecalis* activity). Figure 3 gives a representative concentration-time profile of folate forms using the 20-mg dose. As many previous studies have shown, circulating folate after oral administration is predominantly this 5-methyl metabolite of leucovorin (approximately 97% presystemic conversion) produced in the intestinal mucosa by enzymatic metabolism of 5-formyl-THF (*10–13*). Therefore, the same nonlinear trends observed in AUC and CPEAK of *L casei* activity are observed in the residual after subtraction of the small amount of *S faecalis* activity (not shown). Also notable in figure 3 is the considerably earlier appearance of *S faecalis* activity in the circulation, showing a TPEAK of 40 minutes vs. 2 hours for the major folate peak (vide infra).

In marked contrast to the nonlinear or saturable behavior of serum *L casei* activity, the *S faecalis* component displayed overall linear kinetics through 200 mg (fig. 4). ANOVA pairwise comparison of the dose-adjusted AUC means (table 3) revealed no statistically significant devia-

TABLE 1.—AUC of total folates (*L casei* activity) with increasing leucovorin dose

Dose, mg	No.	Mean AUC[0 – ∞], ng·hr·ml^{-1}	Relative bioavailabilities vs 20 mg PO	vs 200 mg IV	Dose-adjusted mean[a]	SE	Pairwise comparison[b]
20	24	2,184	(100%)	98%	109.2	7.0	$P<0.01$
40	16	3,408	78%	76%	85.2	5.3	$P<0.01$
60	16	4,056	62%	61%	67.6	5.3	$P<0.01$
80	24	4,288	49%	48%	53.6	3.8	NS[c]
100	16	4,600	42%	41%	46.0	3.7	
200 po	6	6,848	31%	31%	34.2	5.0	$P<0.01$
200 iv[d]	6	22,298[e]	—	(100%)	111.5	6.9	

---

[a] Derived by dividing AUC[0 – ∞] by dose.

[b] Using *t*-test on least-squares means that ANOVA MSE as error term. Overall ANOVA $P<0.01$ for 20–100 mg dose comparison.

[c] NS = not statistically significant.

[d] iv dose.

[e] Adjusted by extrapolating from the first hour concentrations (20, 40, 60 min) to an expected CPEAK occurring at 5 min.

TABLE 2.—CPEAK of total folates (*L. casei* activity) with increasing leucovorin dose

Dose, mg	No.	Mean CPEAK, ng/ml[a]	Relative bioavailability vs. 20-mg dose	Dose-adjusted mean	SE	Pairwise comparison[b]
20	24	318	(100%)	15.9	0.90	$P<0.01$
40	16	501	79%	12.5	0.89	$P<0.01$
60	16	574	60%	9.6	0.72	$P<0.01$
80	24	608	48%	7.6	0.56	$P=0.05$
100	16	619	39%	6.2	0.54	

---

[a] Values in μM units are, respectively, 0.67, 1.06, 1.21, 1.28, and 1.31.

[b] Using *t*-test on least-squares means with ANOVA MSE as error term. Overall ANOVA $P<0.01$.

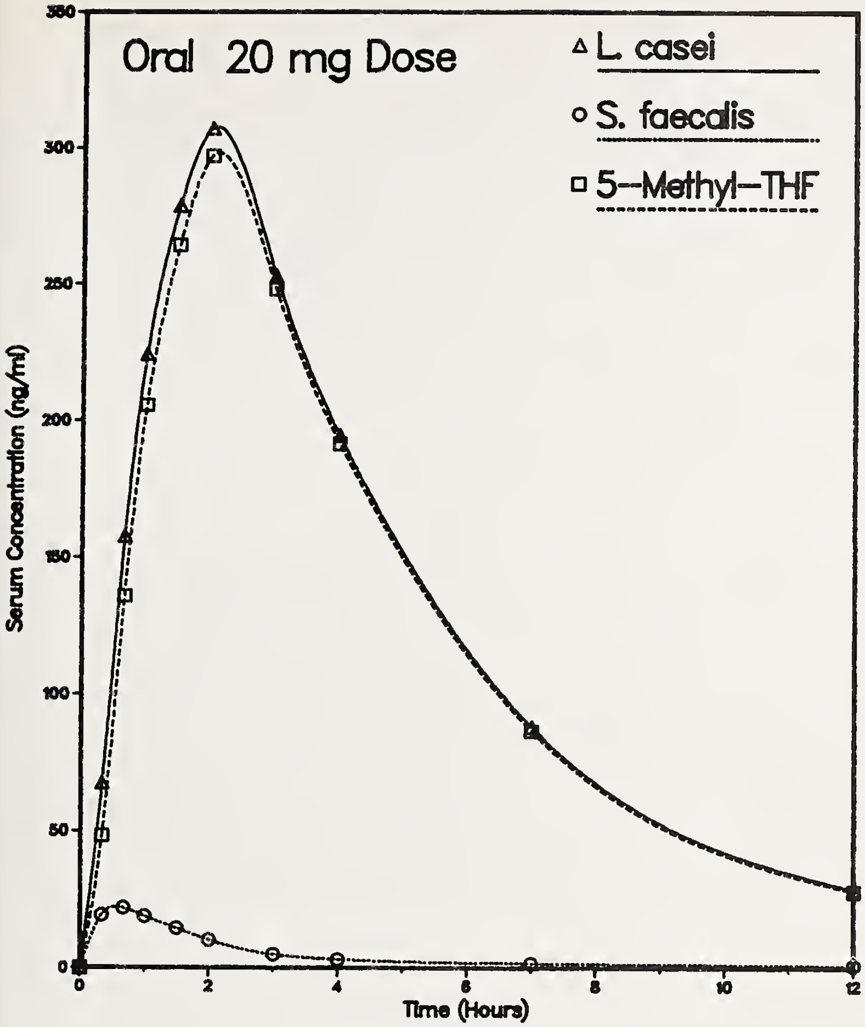


FIGURE 3.—Folate concentration-time profile (ng/ml) following 20-mg dose of leucovorin by differential microbiological assay (24 hr concentrations not shown). (△) *L casei* activity, or total folates; (○) *S faecalis* activity; (□) the difference between *L casei* and *S faecalis* activities, corresponding to 5-methyl-THF.

tion from linearity in this folate fraction with increasing dose. The consequence of this trend, illustrated in table 4, is a consistent increase in the relative contribution of unmetabolized compound to total circulating folates with increasing dose: from about 3% at 20 mg, in agreement with others (*4,12,13*), to almost 10% at 200 mg (*see* Discussion).

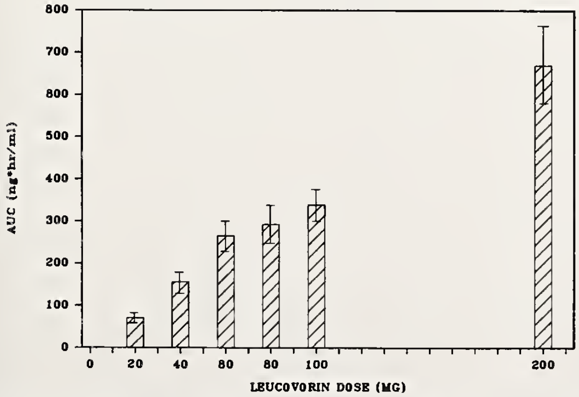


FIGURE 4.—*S faecalis* activity AUC[0 – ∞] (ng·hr·ml^{-1}) for leucovorin doses between 20 and 200 mg. Bars indicate standard error of the mean.

High-Dose Comparison

A more extensive pharmacokinetic characterization was carried out in the substudy employing iv and oral administration of a 200-mg dose. Table 5 presents the key pharmacokinetic parameters of total serum folate with statistical comparison. As expected, AUC[0–24], AUC[0 – ∞], and CPEAK were significantly less for the oral dose ($P<0.01$) while TPEAK was significantly longer. Interestingly, the oral TPEAK (200 min) was also significantly longer than those of the lower oral doses (e.g., 120 min at the 20-mg dose level, $P<0.01$). As previously stated, bioavailability of a 200-mg oral dose of leucovorin was 31% compared with the iv dose (table 1).

The folate concentration-time profiles comparing iv and oral administration, shown in figures 5A and B, highlight the disparities in kinetics. The initial sharp rise in total folate activity following the iv dose (panel A) is almost exclusively the parent drug 5-formyl-THF, as measured by *S faecalis*. This is succeeded by the delayed, gradual rise and prolonged presence of circulating 5-methyl-THF. This appearance of the metabolite has been shown by others to represent a displacement of tissue folates rather than a direct conversion of parenterally administered leucovorin (*10*). The smaller rise in 5-methyl-THF following the oral

TABLE 3.—AUC of *S faecalis* activity with increasing dose

Dose, mg	No.	Mean AUC[0−∞], ng·hr·ml^{-1}	Relative bioavailability vs. 20-mg dose	Dose-adjusted mean[a]	SE	Pairwise comparison[b]
20	23	70.8	(100%)	3.54	0.42	NS[c]
40	15	156.4	110%	3.91	0.70	NS
60	16	265.8	125%	4.43	0.57	NS
80	23	292.8	103%	3.66	0.58	NS
100	16	339.0	96%	3.39	0.39	
200 po	6	669	95%	3.35	0.46	$P<0.01$
200 iv[d]	6	11,367[e]	—	56.83	4.52	

[a] Derived by dividing AUC[0−∞] by dose.

[b] Using *t*-test on least-squares means with ANOVA MSE as error term. Overall ANOVA *P*-value NS for 20–100 mg dose comparison.

[c] NS = not statistically significant.

[d] iv dose.

[e] Adjusted by extrapolating from the first hour concentrations (20, 40, 60 min) to an expected CPEAK occurring at 5 min.

dose (panel B; note different scale) is in contradistinction due to bioconversion of the leucovorin (*10–13*) which occurs at the time of intestinal mucosal transport (*13*). The exact identity of *S faecalis* activity resulting from the oral dose is not unambiguous (see Discussion).

Total folate THALF, VD$_\beta$, and Cl were all found to be independent of the route of administration (table 5). The THALF values of 5.74 and 5.97 hours for iv and oral, respectively, were not significantly different. After correcting for the fraction of oral dose absorbed (see Methods), total folate VD$_\beta$ and Cl were likewise not significantly different between treatments. VD$_\beta$ based on the iv data was 38.4 liters; Cl of total folate was 76.1 ml/minute. Total clearance of the *S faecalis* fraction was 151 ml/minute, twice that of total folate, and was likewise the same for iv and oral (not shown). THALF of *S faecalis* activity, at 3.5 hours for both iv and oral, was shorter than that for total folate. This more rapid removal of unmetabolized compound as reflected in Cl and THALF is reflective of its uptake and replacement by 5-methyl-THF.

TABLE 4.—Serum folate activities as a proportion of total folate AUC

Oral dose, mg	Percent of total folate (*L casei*) activity	
	S faecalis activity (%)	5-Methyl-THF[a] (%)
20	3.2	96.8
40	4.6	95.4
60	6.5	93.5
80	6.8	93.2
100	7.6	92.4
200	9.8	90.2

[a] *L casei* activity minus *S faecalis* activity.

Table 6 summarizes the urinary excretion data from the high-dose study. Folate excretion was roughly proportional to blood levels, as most of that recovered in the urine following the iv dose appeared within the first 2 hours, while the majority of excreted folate from the oral dose appeared between 4 and 12 hours. By 24 hours (the duration of urine collection), 83 mg of biologically active folates (or 83% of the effective dose) were recovered in the urine following iv administration, 31 mg as 5-methyl-THF. In contrast, only 20.2 mg of the oral dose was excreted (representing 65% of the dose actually absorbed), 16 mg as 5-methyl-THF. Comparing Cl and Cl$_R$ between treatments (tables 5 and 6), renal mechanisms accounted for more of the total clearance of the iv dose than that of the oral dose (65.2 vs. 49.1 ml/min, $P=0.05$), although total clearances were the same (76.1 ml/min).

Finally, table 7 illustrates an interesting carry-over phenomenon from treatment to treatment which has not previously been reported. In an analysis of the baseline folate

TABLE 5.—Pharmacokinetic parameters of total folates (*L casei* activity) after iv and oral administration of 200 mg leucovorin

Parameter	iv		Oral		Ratio oral:iv	ANOVA, *P*-value
	Mean	SE	Mean	SE		
AUC[0–24](ng·hr·ml^{-1})	21,667	1,381	6,470	985	0.30	<0.01
AUC[0–∞](ng·hr·ml^{-1})	22,298	1,378	6,848	1,009	0.31	<0.01
CPEAK (ng/ml)	12,829[a]	1,205	859	112	0.07	<0.01
CPEAK (μM)	(27.1)	(2.54)	(1.82)	(0.24)	—	—
TPEAK (min)	5[b]	—	200	12	40	<0.01
K$_{EL}$ (hr^{-1})	0.125	0.01	0.118	0.007	1.06	NS[c]
THALF (hr)	5.74	0.48	5.97	0.35	1.04	NS
VD$_\beta$ (liters)	38.4	5.2	39.9	4.4	1.04	NS
Cl (ml/min)	76.14	4.56	76.11	4.57	1.00	NS

[a] Value extrapolated to the expected peak occurring at 5 min, or the endpoint of the iv infusion. Actual value at first sampling (20 min) was 9,003 ng/ml.

[b] Theoretical peak concentration occurred at a TPEAK of 5 min, the duration of iv infusion. Actual sampling interval was 20 min post-dose.

[c] NS = not statistically significant.

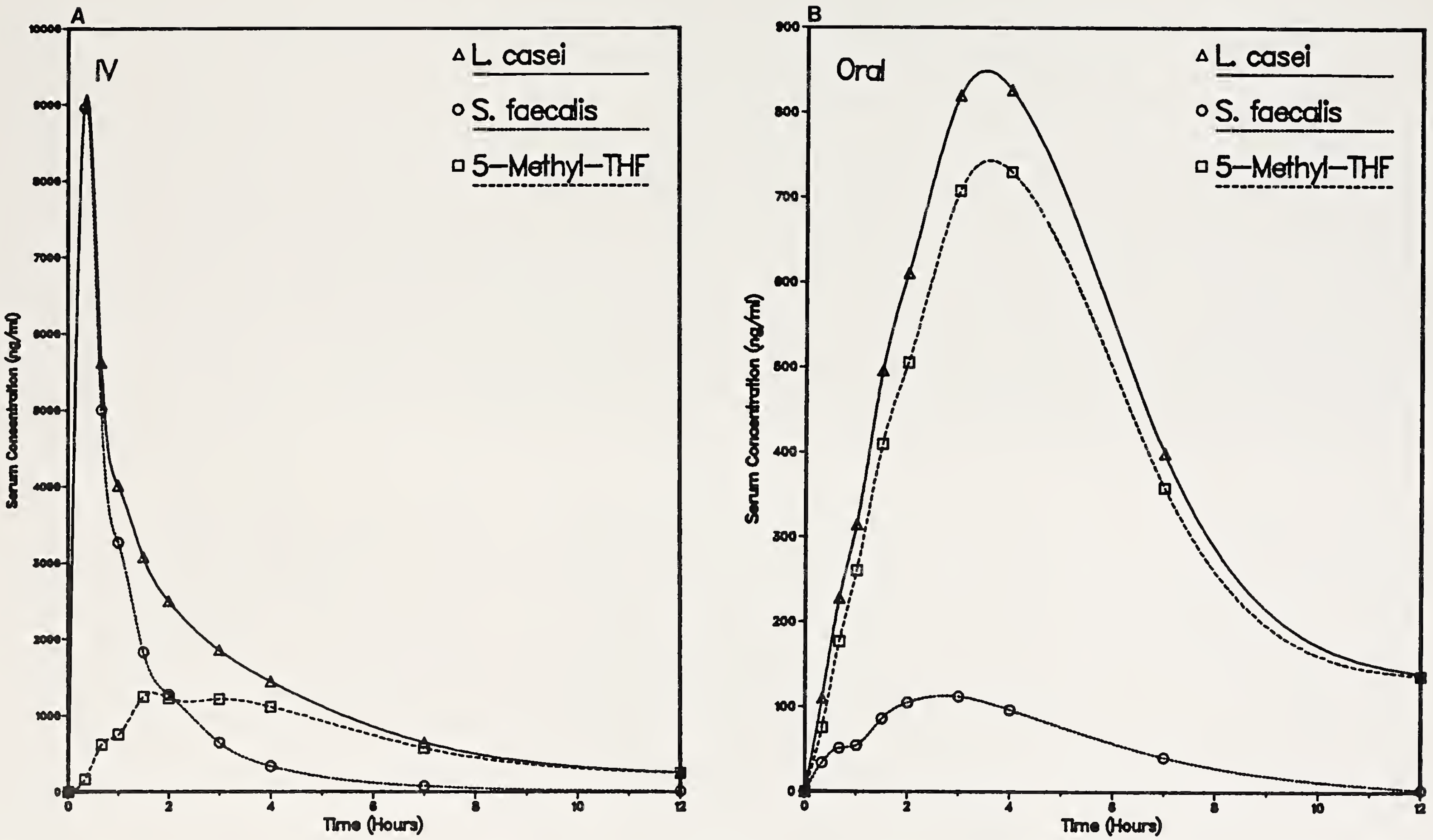


FIGURE 5.—Folate concentration-time profiles (ng/ml) by differential microbiological assay following high-dose (200 mg) leucovorin given iv (A), and orally (B). (△) = *L casei* activity, or total folates; (○) = *S faecalis* activity; (□) = the difference between *L casei* and *S faecalis* activities, corresponding to 5-methyl-THF. Note the 10-fold difference in scale between panels A and B.

TABLE 6.—Urinary excretion of folates after 200-mg leucovorin administration

	Cumulative excreted folate, mg					
	0–2 hr	2–4 hr	4–12 hr	12–24 hr	% Dose excreted[a]	Cl_R, ml/min
Iv dose:						
Total folates[b]	45.7	61.9	80.2	82.9	83[c]	65.2[d]
5-Methyl-THF[e]	6.1	13.8	28.2	30.8	—	50.0[f]
Oral dose:						
Total folates[b]	1.9	6.5	18.5	20.2	20[c]	49.1[d]
5-Methyl-THF[e]	1.3	5.1	15.2	16.6	—	44.9[f]

[a] Based on biologically active (l-isomer) percent of dose, or 100 mg.
[b] *L casei* activity.
[c] $P<0.01$.
[d] $P=0.05$.
[e] *L casei* minus *S faecalis* activities.
[f] Data were not significantly different.

means for each of the 4 study periods, it was found that 1 week after a single dose of leucovorin, plasma folate levels were still detectably elevated. The initial mean baseline of *L casei* activity for all 24 subjects (i.e., prior to any dose) was 5.4 ng/ml (11 n*M*). One week after the first treatment the mean baseline for the second period was almost double that at 10.3 ng/ml ($P=0.04$). Each successive baseline value likewise increased over that preceding. This effect was even more marked with the 200-mg dose used in the 6-subject study, in which the *L casei* baseline mean more than tripled from 4.1 to 15.1 ng/ml ($P<0.01$) between periods 1 and 2. This was not observed in the *S faecalis* activity (not shown). All analyses in these studies were performed on reduced data (i.e., post-dose concentration minus baseline) to minimize such confounding carry-over effects. Conceivably, however, many aspects of folate metabolism, clearance, excretion, etc., would be influenced by previous loading with leucovorin doses such as these. This carry-over phenomenon is indicative of repletion of the body's folate stores by the administered leucovorin and of retention for a sustained time in a deep-tissue pool.

DISCUSSION

This dose-proportionality investigation of orally administered leucovorin calcium ((d,l)-5-formyl-THF) in healthy male subjects clearly demonstrated a significant deviation from linearity in AUC and CPEAK of circulating total folates with increasing doses consistent with a saturation of intestinal transport of folate. The lowest dose studied (20 mg) served as a reliable estimate of complete bioavailability based on its comparability to an intravenous dose and to previous reports (*4,12,14*). Incrementally higher doses revealed a marked failure to proportionally increase circulating folate levels through the 100-mg dose, which itself displayed a relative bioavailability of 42%. These observations were borne out in the separate comparison of a 200-mg dose administered intravenously and orally, the latter giving only a 31% absolute bioavailability relative to the former.

TABLE 7.—Mean predose (baseline) total folate levels (*L casei* activity) for each treatment period

	Mean baseline, ng/ml	SE	Pairwise comparison[a]
Dose-proportionality ($n=24$)			
Period I	5.4	0.7	$P=0.04$
Period II	10.3	1.2	$P=0.05$
Period III	13.3	1.5	NS[b]
Period IV	14.9	1.6	
High-dose group ($n=6$)			
Period I	4.1	0.6	$P<0.01$
Period II	15.1	2.5	

[a] Overall ANOVA significance of period effect was <0.01 for 20–100 mg dose study.
[b] NS = not statistically significant.

That the early *S faecalis* component displayed in contrast linear or dose-proportional kinetics is suggestive of uptake independent of the saturable carrier-mediated intestinal transport system. Its early rise and peak, lack of conversion to 5-methyl-THF, and nonsaturable kinetics may be explained by a passive diffusion of a small amount of 5-formyl-THF proximal to the jejunum, thereby bypassing intestinal transport and presystemic metabolism.

As the dose increased, serum *S faecalis* activity increased in proportion to that of *L casei* and also appeared later than at lower doses (compare figures 3 and 5B), almost as a second rise. At the 200-mg dose, *S faecalis* was nearly 10% of *L casei* activity, peaking at 3 hours, just slightly prior to the total folate peak. The relative decrease in the metabolite 5-methyl-THF indicated by those activities intimates that the bioconversion process itself may be saturable, since relatively more of the folate was being absorbed unmetabolized. This observation supports that made by others that intestinal transport is independent of intestinal metabolism (*13,15*). It is also noteworthy that the conversion of 5-formyl-THF to 5-methyl-THF is not a single step but requires at least 3, including loss of formyl group (*12*), reacquisition of a carbon moiety, and reduction.

As alluded to previously, the specific folate form(s) which *S faecalis* measured is not definitive, and this activity is not necessarily synonymous with l-5-formyl-THF. This organism is responsive to all (l)-pteroylmonoglutamates except 5-methyl-THF (see Methods); these include THF and both the 10- and 5-isomers of formyl-THF, the latter of which is known to nonenzymatically cyclodehydrate to 5,10-methenyl-THF at low pH (*12*). The folate measured by *S faecalis* following parenteral leucovorin administration is most certainly the parent compound 5-formyl-THF. Perry and Chanarin (*10*) suggested that the small amount of *S faecalis* activity following oral doses was pteroylglutamate (oxidized folate). However, Pratt and Cooper (*11*) and Whitehead et al. (*13*) chromatographed 20- to 30-minute blood samples and identified the *S faecalis* activity as 5-formyl-THF. Contrary to both of these, in the double-isotope studies of Nixon and Bertino (*12*), a later (75 min) blood sample contained no parent compound, but 9% of the total folate chromatographed as either 10-formyl- or 5,10-methenyl-THF. Nevertheless, they did report 40% of that excreted in the urine to be 5-formyl-THF. It is likely in the present investigation that the very early (i.e., 20–60 min) *S faecalis* form is unmetabolized parent compound; that appearing later (see figure 5B) may be a mixture of folates at the formyl level of oxidation, or nonmethylated THF.

The folate THALF values calculated from these data were considerably longer than the half-lives previously reported by others (*4,16,17*). Based on the sensitive microbiological assay used in this study, plasma folates from the leucovorin administration were well above quantifiable limits through 24 hours, and after baseline adjustment were still elevated. Our THALF, derived from the elimination rate constant at the 7- to 24-hour interval, therefore represented the true terminal half-life of the elimination phase rather than the earlier distribution phase. This high assay sensitivity also allowed the detection of the small (but statistically significant) elevation in circulating folates still present 1 week after the study dose. The even longer elimination phase and the putative mechanism of this folate retention (liver, RBC, biliary recycling, etc.) await elucidation.

Beyond the use of leucovorin calcium in folate deficiencies and the present utilization for methotrexate rescue, current clinical research is exploring the interaction of reduced folates with the activated form of 5-fluorouracil, 5-fluorodeoxyuridylate (5FdUMP) (*18*). The target enzyme of this cytotoxic drug is thymidylate synthetase, which functions in de novo pyrimidine biosynthesis (*19*). A series of cell culture investigations has defined 1×10^{-5} M (10μM) as the "benchmark" of reduced folates required to effect the desired drug potentiation (*20,21*). As normal plasma folate levels are in the range of 6 to 20 ng/ml (*22*), or 13 to 43 nM, exogenous supplementation is clearly required. Figures 5A and B demonstrate that a 200-mg oral dose does not achieve the theoretical 10 μM (4700 ng/ml) requirement of reduced folates, while the same iv dose well exceeds it. Further adjustment of the duration of iv infusion and the dosage would allow the maintenance of that concentration for the 4 hours thought to be optimal (*17*). It should, however, be pointed out that concentrations in the plasma do not necessarily equate with those in the cytoplasm, the site of thymidylate synthetase activity and of any putative interaction. The specific THF coenzyme which participates in the ternary complex of 5FdUMP, enzyme, and folate is 5,10-methylene-THF, an intracellular cyclized derivative (*21,23,24*). Under normal circumstances, all forms of THF are freely interconvertible, the important factor being the magnitude of the total reduced folate pool. Consequently, emphasis in this study has been on the pharmacokinetics of total plasma (l)-folates after leucovorin administration, as 5-methyl- and 5-formyl-THF are normally equally active biologically in generating the particular folate coenzymes. Work is currently underway to quantitate in these biological samples, using high-performance liquid chromatography, the inactive (d)-diastereoisomer which constitutes half of the administered dose of leucovorin.

These pharmacokinetic data together with the growing body of information on human folate metabolism should aid the clinician in the development of rational dosages and schedules for leucovorin calcium in the many cancer chemotherapeutic regimens in which it is utilized.

REFERENCES

(*1*) HEPNER GW, BOOTH CC, et al: Absorption of crystalline folic acid in man. Lancet 2:302–306, 1968.

(*2*) COOPER BA: Physiology of absorption of monoglutamyl folates from the gastrointestinal tract. *In* Folic Acid (Broquist HP, Butterworth CE, Wagner C, eds). Washington, DC: National Academy of Sciences, 1977, pp 188–197.

(*3*) BERTINO JR, NIXON PF, NAHAS A: Mechanism of uptake of folate monoglutamates and their metabolism. *In* Folic Acid (Broquist HP, Butterworth CE, Wagner C, eds). Washington, DC: National Academy of Sciences, 1977, pp 178–187.

(*4*) STRAW JA, SZAPARY D, WYNN WT: Studies on the pharmacokinetics of the diastereoisomers of leucovorin after intravenous and oral administration to normal subjects. Cancer Res 44:3114–3119, 1984.

(*5*) ROBERTS EC, SNELL EE: An improved medium for microbiological assays with *Lactobacillus casei.* J Biol Chem 163:499, 1946.

(*6*) WATERS AH, MOLLIN DL: Studies of the folic acid activity of human serum. J Clin Pathol 14:335, 1961.

(*7*) TAMURA T, SHIN YS, WILLIAMS MA, et al: *Lactobacillus casei* response to pteroylglutamates. Anal Biochem 49(2): 517, 1972.

(*8*) WILSON SD, HORNE DW: Use of glycerol-cryoprotected *Lactobacillus casei* for microbiological assay of folic acid. Clin Chem 28:1198, 1982.

(*9*) COSSINS EA: Folates in biological materials. *In* Folates and Pterins; vol I (Blakley RL, Benkovic SJ, eds). New York: John Wiley & Sons, 1984, pp 1–59.

(*10*) PERRY J, CHANARIN I: Intestinal absorption of reduced folate compounds in man. Br J Haematol 18:329–339, 1970.

(*11*) PRATT RF, COOPER BA: Folates in plasma and bile of man after feeding folic acid-^{3}H and 5-formyltetrahydrofolate (folinic acid). J Clin Invest 50:455–462, 1971.

(*12*) NIXON PF, BERTINO JR: Effective absorption and utilization of oral formyltetrahydrofolate in man. N Engl J Med 286:175–179, 1972.

(*13*) WHITEHEAD VM, PRATT R, VIALLET A, et al: Intestinal conversion of folinic acid to 5-methyltetrahydrofolate in man. Br J Haematol 22:63–72, 1972.

(*14*) LASSETER KC, SHAMBLEN E, WOODWARD D, et al: Bioavailability of oral and parenteral formulations of leucovorin. Clin Pharmacol Ther 33:222, 1983.

(*15*) COOPER BA: Folate—its metabolism and utilization. Clin Biochem 17:95–98, 1984.

(*16*) MEHTA BM, GISOLFI AL, HUTCHISON DJ, et al: Serum distribution of citrovorum factor and 5-methyltetrahydrofolate following oral and im administration of calcium leucovorin in normal adults. Cancer Treat Rep 62:345–350, 1978.

(*17*) CAMPBELL J, RUSTUM YM, CANOBBIO L, et al: Metabolic modulation of 5-fluorouracil (FU) by thymidine (TdR) and citrovorum factor (CF). Proc 13th Int Congr Chemother 221:17–22, 1983.

(*18*) MACHOVER D, SCHWARZENBERG L, GOLDSCHMIDT E, et al: Treatment of advanced colorectal and gastric adenocarcinomas with 5-FU combined with high-dose folinic acid; a pilot study. Cancer Treat Rep 66(10):1803–1807, 1982.

(*19*) SANTI DV, DANENBERG PV: Folates in pyrimidine nucleotide biosynthesis. *In* Folates and Pterins, vol I (Blakley RL, Benkovic SJ, eds). New York: John Wiley & Sons, 1984, pp 345–398.

(*20*) EVANS RM, LASKIN JD, HAKALA MT: Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. Cancer Res 41:3288–3295, 1981.

(*21*) HOUGHTON JA, MARODA SJ, PHILIPS JO, et al: Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinomas in mice. Cancer Res 41:144–149, 1981.

(*22*) WORLD HEALTH ORGANIZATION: Nutritional Anaemias. WHO Tech Rep Ser No. 503. Geneva: World Health Organ, 1972.

(*23*) Danenberg PV, Lagenbach RJ, Heidelberger C: Structures of reversible and irreversible complexes of thymidylate synthetase and fluorinated pyrimidine nucleotides. Biochemistry 13:926–933, 1974.

(*24*) Ullman B, Lee M, Martin DW, et al: Cytotoxicity of 5-fluoro-2′-deoxyuridine: Requirement for reduced folate cofactors and antagonism of methotrexate. Proc Natl Acad Sci USA 75:980–983, 1978.

Bioavailability of High-Dose Oral Leucovorin

John D. Hines,[1,*] Michael H. Zakem,[1] David J. Adelstein,[1] Paul Giroski,[1] M. Robert Blum,[2] and Youcef M. Rustum[3,4]

ABSTRACT—Fifteen adult subjects comprised the study group; 2 were colon cancer patients. Total leucovorin (citrovorum factor; CF) doses of 200, 400, 800, and 1600 mg were equally subdivided and administered at 0, 1, 2, and 3 hours. Three of the subjects were fasting and the other 12 were not. Quantitation of serum L-CF by *Pediococcus cerevisiae* and total reduced folates (TRF) by radioassay were performed in 10 samples from each subject drawn over a 12-hour period after initiation of CF dosing. The mean serum levels of L-CF remained >0.1 μM at 3 hours and the TRF 3.5 μM at 5 hours, respectively, after termination of CF dosing in all subjects treated at the 800- and 1600-mg dose schedule. At all CF dosage schedules employed, peak serum concentrations were reached within 4 and 6 hours after initiation of oral CF. Peak TRF concentrations at the 200-, 400-, 800-, and 1600-mg doses were 3.42 ± 0.65, 4.05 ± 1.04, 4.81 ± 0.14, and 5.11 ± 1.81 μM, respectively. Peak L-CF levels at 200, 400, 800, and 1600 were 0.15 ± 0.11, 0.21 ± 0.14, 0.23 ± 0.11, and 0.34 ± 0.16 μM. Based upon these observations, the following conclusions were reached: 1) no significant differences were observed in serum concentrations of folates between the 800- and 1600-mg dose schedules; 2) at these doses serum concentrations of L-CF and TRF were achieved that warrant a phase I investigation of high-dose oral CF with standard dose FUra in patients with advanced colorectal carcinoma—NCI Monogr 5:57–60, 1987.

The biochemical mechanism by which the enzyme dTMP synthetase is inhibited by 5-fluorodeoxyuridine monophosphate (FdUMP) has permitted the theoretic potential utility of FUra in combination with CF in the treatment of colorectal carcinoma. In the presence of intracellular reduced folate, a ternary complex of dTMP synthetase and FdUMP accumulates, which results in a potent inhibition of dTMP synthetase in colon carcinoma cells (*1–4*). In the absence of the folate cofactor, FdUMP forms a weak binary complex with dTMP synthetase [dissociation constant (K_d) = 10^{-5} M]. However, in the presence of intracellular reduced folate, a resultant covalent ternary complex forms in which FdUMP is more tightly bound ($K_d = 10^{-12}$ M) (*5*). Logically, the coadministration of L-CF with fluorinated pyrimidines would effect enhanced inhibition of dTMP synthetase by FdUMP. This hypothesis has now been tested and verified in a number of laboratories utilizing L1210 cells in tissue culture and in colon cancer cells derived from mouse xenografts (*5,6*).

A number of clinical studies employing FUra and CF in patients with colorectal carcinoma have now been reported. Rustum and co-workers have documented complete and partial remissions in patients with colorectal carcinoma who have either been refractory to prior FUra or have received no prior chemotherapy (*7*). The therapeutic regimen which they utilized was a 2-hour infusion of 500 mg/m^2 of CF with an iv bolus of FUra, 600 mg/m^2, given 1 hour after the start of CF infusion.

Studies at Cleveland Metropolitan General Hospital have confirmed and extended these results utilizing the same 2-hour infusion of CF with bolus administration of 600 mg/m^2 of FUra at 1 hour (*8*). Other studies employing continuous infusion of CF and FUra have resulted in varying degrees of clinical efficacy both in untreated patients and in patients previously treated with FUra (*9–11*).

The high cost of employing high-dose iv CF becomes a prohibitive factor in contemplating large-scale cooperative group trials. The potential for production and utilization of less costly oral CF prompted this study which explores the bioavailability of high-dose oral CF in adult subjects with and without antecedent colon carcinoma.

MATERIALS AND METHODS

Fifteen adult subjects comprised the study group. The ages ranged from 26 to 65 years old (median age = 42). There were 6 females and 9 males. The weights ranged from 52 to 76 kg. Two patients, who were in clinical remission, had prior surgery for Dukes B colon carcinoma 2 and 5 years previous to the study. The remaining 13 were normal volunteer control subjects. Informed signed consent was obtained in all study subjects. None of the 15 had any prior gastric or small intestinal surgery or history suggestive of small bowel malabsorption. The total oral CF doses (supplied as Wellcovorin tablets of 25 mg each) were administered in equally divided doses at 0, 1, 2, and 3 hours. Eleven subjects were entered into only 1 leg of the study. One subject received leucovorin at all 4 dose levels. Three received leucovorin at 2 dose levels. Although no attempt was made to keep the subjects fasting, 3 of the normal volunteers remained fasting except for water during the 4-hour L-CF ingestion. No other medications were ingested either 12 hours preceding or during the study period. The total doses of CF administered were 200 mg, 400 mg, 800 mg, and 1600 mg. Blood samples for analysis of L-CF and total reduced

---

ABBREVIATIONS: AUC = area under curve; CF = leucovorin (citrovorum factor); C_{max} = peak concentration; FdUMP = 5-fluorodeoxyuridine monophosphate; K_d = dissociation constant; T_{max} = time of peak; TRF = total reduced folates.

---

[1] Division of Hematology/Oncology, Cleveland Metropolitan General Hospital, Case Western Reserve University, OH.

[2] Burroughs Wellcome Company, Research Triangle Park, NC.

[3] Roswell Park Memorial Institute, Buffalo, NY.

[4] We thank Mrs. Anne LaGania for secretarial assistance.

* *Reprint requests to:* John D. Hines, M.D., Division of Hematology/Oncology, Cleveland Metropolitan General Hospital, 3395 Scranton Road, Cleveland, OH 44109.

TABLE 1.—Bioavailability parameters for L-CF[a]

Dose, mg	N	Cmax, μM	Tmax, hr	AUC, $\mu M \times$hr
200	5	0.15±0.11	3.6±1.8	0.67±0.57
400	4	0.21±0.14	2.4±1.9	0.97±0.76
800	6	0.23±0.11	4.3±0.3	1.17±0.50
1600	6	0.33±0.16	4.7±0.8	1.82±1.04

[a] Mean±SD

folates were drawn at 0, 0.5, 1, 4, 4.5, 5, 5.5, 6, 8, 10, and 12 hours after commencing oral leucovorin ingestion.

Serum samples for L-CF and TRF were assayed in triplicate. Quantitative serum L-CF concentrations were determined by microbiologic assay employing *Pediococcus cerevisiae* (ATCC 8081). TRF were quantitated by the radioligand assay of Kamen et al. (*12*). Additionally, serum folate concentrations were determined by microbiologic assay with *Lactobacillus casei (13)*. In selected serum samples, separation and quantitation of monoglutamate folate derivatives were determined by a high-pressure liquid chromatographic reverse phase ion-pair technique (*14*). Areas under the curve (AUC) for L-CF and TRF were determined by the normal trapezoidal rule method. Peak concentrations (Cmax) and time of peak (Tmax) were observed values.

RESULTS

The principle bioavailability parameters (Cmax, Tmax, AUC) for the varying high-dose CF regimens are presented in table 1. Mean (±SD) L-CF concentration-time curves for the 800- and 1600-mg regimens are shown in figures 1 and 2. Although there is a relatively large standard deviation at each dose level, the 3 fasting subjects exhibited the highest serum L-CF levels, which were all within a much narrower standard deviation (not depicted). The mean serum levels of L-CF remained <0.35 μM for all regimens at all times.

Table 2 presents the bioavailability parameters for the serum TRF pools. The mean (±SD) TRF concentration-time curves for the 800- and 1600-mg regimens are shown in figures 3 and 4. The variability in TRF levels, as indicated by coefficient of variation of AUC, was considerably less than for L-CF levels. In the 3 fasting subjects, the peak serum values and AUC were higher with a significantly smaller SD as contrasted with the remaining subjects. The mean serum levels of TRF remained >3.0 μM up to 7 hours after termination of oral leucovorin administration in the 800- and 1600-mg regimens. TRF serum profiles shown in figure 5 illustrate the composite levels in the 1 subject who received all 4 dose levels. For this subject, increased levels with increasing dose (although not dose-proportional) are seen from 200 to 1600 mg.

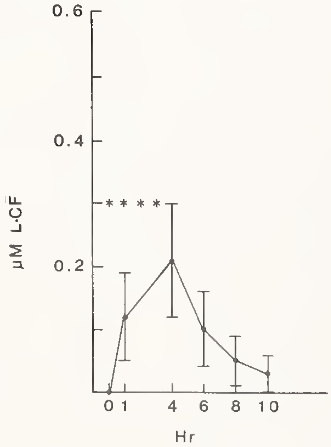


FIGURE 1.—Serum concentration of L-CF±SD after the 800-mg oral CF dose. * = 200-mg ingestion; ● = average serum concentration.

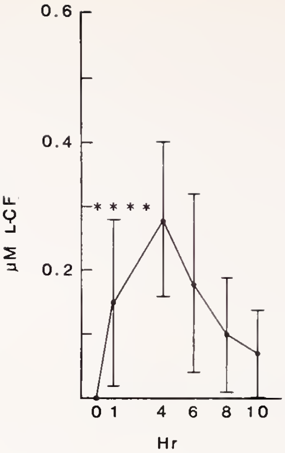


FIGURE 2.—Serum concentration of L-CF±SD after the 1600-mg oral CF dose. * = 400-mg ingestion; ● = average serum concentration.

Random testing of serum samples obtained at 4 and 6 hours were performed for folate monoglutamate derivatives using high-performance liquid chromatography. 5-Methyltetrahydrofolate levels did not exceed 4.0 μM in either the 800-mg or the 1600-mg leucovorin dose regimen.

Analysis of AUC data for L-CF and TRF (tables 1 and 2) revealed a statistically significant difference for the L-CF values between the 200- and 1600-mg dose schedules (P=.02). Near significance is observed for the TRF levels between the 200- and 800-mg (P=.07) and the 200- and 1600-mg (P=.06) schedules. The lack of statistical significance reflects the small number of subjects in the study. Although this pilot study was not designed to have the power for statistical analysis, the trend to increased levels of both L-CF and TRF is clear up to the 800-mg leucovorin dose level.

DISCUSSION

In early clinical trials, Bruckner and co-workers demonstrated the clinical efficacy of oral L-CF administered thrice daily with simultaneous iv FUra infusion in colon cancer patients who were refractory to the same dose schedule of FUra administered as a single agent (*15*). Subsequent published studies, employing a variety of dosage schedules of

TABLE 2.—Bioavailability parameters for TRF[a]

Dose, mg	N	Cmax, μM	Tmax, hr	AUC, $\mu M \times$hr
200	4	3.42±0.63	4.7±0.6	24.1±6.8
400	4	4.05±1.03	6.5±1.8	32.3±11.8
800	6	4.81±1.20	6.4±1.3	39.9±12.0
1600	6	5.11±1.81	5.8±1.7	40.4±16.3

[a] Mean±SD

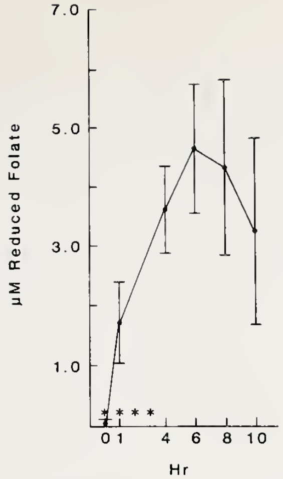


FIGURE 3.—Serum concentration of TRF after the 800-mg oral CF dose. *See* fig. 1 legend.

high-dose iv CF coupled with either bolus or continuous infusion of FUra, have demonstrated an even higher clinical response in untreated patients with advanced colorectal carcinoma (*7-11,15*). Preliminary data from a recently completed phase III study from Roswell Park Memorial Institute clearly demonstrated a >40% response rate with high-dose iv CF (500 mg/m^2) in a 2-hour infusion with bolus FUra (600 mg/m^2) administered at 1 hour for 6 weeks. This arm of the study was clinically superior to the FUra plus methotrexate arm and was associated with acceptable toxicity (Petrelli N, Herra L, Stule J, et al, submitted for publication). We have recently completed a phase I pilot study for the same CF and FUra regimen in 30 evaluable patients with relapsed colorectal carcinoma. Half of the patients had demonstrated measurable disease progression on FUra and the remainder were previously untreated with FUra. The overall complete and partial remission rate was 47% with 27% of patients exhibiting stable disease (Zakem M, Hines JD, Adelstein DJ, et al, submitted for publication). Disease progression was observed only in those patients with prior FUra exposure. Currently, the available published data tend to support the conclusion that high-dose short-term iv CF infusion coupled with bolus iv infusions of FUra exhibit a higher degree of clinical efficacy as compared with continuous infusion of CF with FUra (*7,8,15*). This may be, in part, due to the lower plasma L-CF levels achieved with the latter coupled with the observation that most of the circulating folate is 5-methyltetrahydrofolate and not L-CF.

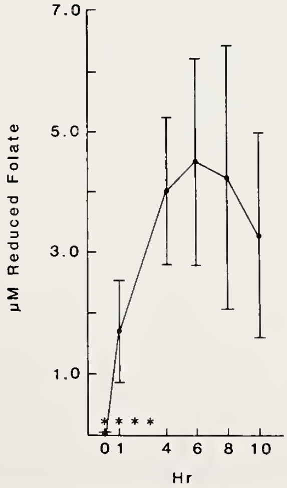


FIGURE 4.—Serum concentration of TRF after the 1600-mg oral CF dose. *See* fig. 2 legend.

The pharmacokinetics of high-dose iv CF have been described by Rustum and co-workers (*7*). In a study employing a 500-mg/m^2 2-hour infusion schedule of CF, the plasma concentration of 5-methyltetrahydrofolate at 5 minutes and 24 hours represented 17% and 37% of the total plasma 5-formyltetrahydrofolate concentration, respectively. Initial results demonstrated a biphasic disappearance of 5-formyltetrahydrofolate, comprising an initial phase with a half-life of nearly 2 hours and a subsequent delay phase with a half-life of nearly 7 hours. Noteworthy is the fact that the active isomer, L-5-formyltetrahydrofolate, was eliminated more rapidly than total drug. While the mean peak of the L-stereoisomer was approximately 10 μM, only 0.2% was detected at 24 hours. In these studies, the high-dose CF was not demonstrated to perturb in any way the plasma pharmacokinetics of the FUra administered. Previous studies by Nixon and Bertino demonstrated that at 90 minutes after an iv bolus of radiolabelled L-CF, an excess of greater than 60% of the radiolabel was present as 5-methyltetrahydrofolate (*16*). In contrast, when the radiolabelled L-CF was administered orally, virtually all the radiolabel was 5-methyltetrahydrofolate. Straw and coworkers substantiated and extended these observations for both iv and oral administration of CF to normal control subjects (*17*). These authors demonstrated the rapid plasma clearance of L-CF at doses ranging from 25 to 100 mg. Additionally, they demonstrated that absorption of both the D-and L-isomers was saturated and the bioavailability was decreased at oral doses of 50 or 100 mg. They also demonstrated that the plasma half-life of 5-methyltetrahydrofolate was significantly prolonged by repeated oral doses of CF. Blum and associates, employing a single oral dose of 15 mg of CF, demonstrated that 100% of the vitamin was bioavailable and was equivalent to serum values obtained after parenteral administration (Blum MR, Guaspari A, Liao FH, et al, this monograph).

The oral dosing schedule of CF utilized in this pilot study was designed to avoid the saturation phenomenon demonstrated in the other studies. This dosage administration also permits a feasible means of oral CF "loading," which can be accomplished in the outpatient clinic.

Based upon the results of this study we conclude the following: 1) no significant differences were observed in serum concentrations of reduced folates between the 400-mg, 800-mg and 1600-mg doses; and 2) at doses exceeding 200 mg (hourly for 4 hours), the serum concentrations of L-CF and TRF reached levels that have been reported to potentiate FUra in vitro (*5,6,18*). Although the peak plasma levels of TRF did not reach 10-μM concentrations, it is possible, based upon the AUC data and duration of sustained plasma folate concentration, that the tumor tissue might well accumulate folate pools ≥10 μM. A clinical phase I-II study of high-dose oral CF with a dosage escalation of FUra in patients with advanced colorectal carcinoma appears warranted.

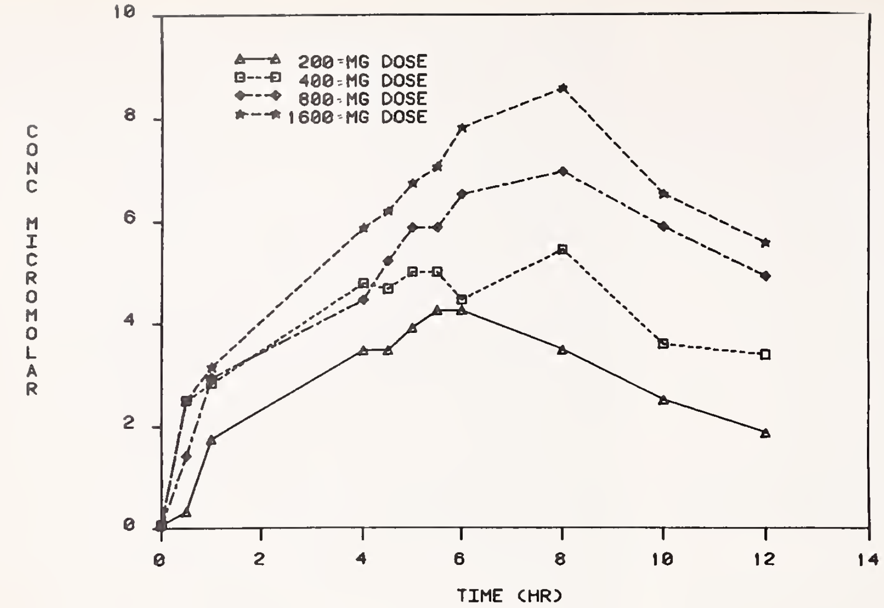


FIGURE 5.—Serum TRF levels following 4 doses of CF in 1 subject.

REFERENCES

(*1*) FRIEDKIN M: Thymidylate synthetase. Adv Endocrinol 38:235–292, 1973.

(*2*) SANTI DV, POGOLOTTI AL, JAMES TL, et al: Thymidylate synthetase: Interaction with 5-fluoro- and 5-trifluoromethyl-2-deoxyuridylic acid. *In* Biochemistry Involving Carbon-Fluorine Bonds (Filler R, ed). Washington, DC: American Chemical Society, 1976, pp 57–76.

(*3*) BENKOVIC SJ, BULLARD WP: On the mechanisms of action of folic acid cofactors. Prog Bioorg Chem 2:135–176, 1973.

(*4*) HEIDELBERGER C: Fluorinated pyrimidines and their nucleosides. *In* Antineoplastic and Immunosuppression Agents (Sartorelli AC, Johns DG, eds), vol 11. New York: Springer-Verlag, 1975, pp 193–231.

(*5*) SANTI DV: A biochemical rationale for the use of 5-fluorouracil in combination with leucovorin. *In* Advances in Cancer Chemotherapy: The Current Status of 5-fluorouracil-Leucovorin in Calcium Combination: Symposium Sponsored by Roswell Park Memorial Institute (Bruckner HW, Rustum YM, eds). New York: John Wiley & Sons, 1984, pp 1–11.

(*6*) HOUGHTON JA, HOUGHTON PJ: Basis for interaction of 5-fluorouracil and leucovorin in colon adenocarcinoma. *In* Advances in Cancer Chemotherapy: The Current Status of 5-Fluorouracil-Leucovorin in Calcium Combination: Symposium Sponsored by Roswell Park Memorial Institute (Bruckner HW, Rustum YM, eds). New York: John Wiley & Sons, 1984, pp 23–32.

(*7*) RUSTUM YM, MADAJEWICZ S, CAMPBELL J, et al: Clinical biochemical pharmacology of 5-formyltetrahydrofolate in combination with 5-fluorouracil in patients with advanced colorectal carcinoma. Correlations with clinical response. *In* Advances in Cancer Chemotherapy: The Current Status of 5-Fluorouracil–Leucovorin in Calcium Combination: A Symposium Sponsored by Roswell Park Memorial Institute (Bruckner HW, Rustum YM, eds). New York: John Wiley & Sons, 1984, pp 77–82.

(*8*) ZAKEM MH, ADELSTEIN DJ, HINES JD, et al: High-dose leucovorin and 5-fluorouracil (5-FU) in refractory and relapsed colorectal carcinoma. Proc ASCO 5:81, 1986.

(*9*) BUKOWSKI RM, CUNNINGHAM J: Efficacy of 5-fluorouracil in combination with leucovorin in patients with colorectal carcinoma: Continuous infusion of low doses. *In* Advances in Cancer Chemotherapy: The Current Status of 5-Fluorouracil–Leucovorin in Calcium Combination: A Symposium Sponsored by Roswell Park Memorial Institute (Bruckner HW, Rustum YM, eds). New York: John Wiley & Sons, 1984, pp 69–74.

(*10*) MACHOVER D, SCHWARZENDERG L, MATHÉ G: Treatment of advanced colorectal and gastric adenocarcinoma with 5FU combined with high-dose folinic acid. A pilot study. Cancer Treat Rep 66:1803–1807, 1982.

(*11*) BYRNE PJ, TREAT J, MCFADDEN M, et al: Therapeutic efficacy of the combination of 5-fluorouracil and high-dose leucovorin in patients with advanced colorectal carcinoma. *In* Advances in Cancer Chemotherapy: The Current Status of 5-Fluorouracil–Leucovorin in Calcium Combination: Symposium Sponsored by Roswell Park Memorial Institute (Bruckner HW, Rustum YM, eds). New York: John Wiley & Sons, 1984, pp 65–68.

(*12*) KAMEN BA: Direct radiochemical assay for serum folate competition between ^{3}H-folic acid and 5-methyltetrahydrofolate for a folate binder. J Lab Clin Med 83:164, 1974.

(*13*) HERBERT V: Aseptic addition method for *Lactobacillus casei* assay of folate activity in human serum. J Clin Pathol 19:12–15, 1966.

(*14*) HORNE DW, BRIGGS WT, WAGNER C: High pressure liquid chromatographic separation of the naturally occurring folic acid monoglutamate derivatives. Anal Biochem 116:393–397, 1981.

(*15*) BRUCKNER HW, STORCH JA, HOLLAND JF: Leucovorin increases the toxicity of 5-fluorouracil: Phase I clinical pharmacologic trials. Proc Am Assoc Cancer Res–ASCO 22:192, 1981.

(*16*) NIXON PF, BERTINO JR: Effective absorption and utilization of oral formyltetrahydrofolate in man. N Engl J Med 286:175–179, 1972.

(*17*) STRAW JA, SZAPARY D, WYNN WT: Pharmacokinetics of the diastereoisomers of leucovorin after intravenous and oral administration to normal subjects. Cancer Res 44:3114–3119, 1984.

(*18*) MINI E, MARSON BA, BERTINO JR: Leucovorin (LV)-fluoropyrimidine synergy in the human leukemia lymphoblast cell line CCRF-CEM. Proc Am Assoc Cancer Res 26:724, 1985.

Biochemical and Pharmacologic Rationale for High-Dose Methotrexate

Jacques Jolivet[1,*]

ABSTRACT—High-dose methotrexate (HDMTX) regimens were initially designed to overcome methotrexate (MTX) resistance due to defective transport of the drug. At high concentrations, enough MTX diffuses into resistant cells to saturate and inhibit the target enzyme, dihydrofolate reductase (DHFR). The high doses of MTX needed to achieve these high drug concentrations must be administered with the reduced folate antidote, leucovorin (LV; 5-formyltetrahydrofolate), to prevent increased toxicity. To increase MTX therapeutic index, LV rescue must be selective, i.e., more effective in normal than in tumor cells. In experimental models, selective rescue can be achieved if strict LV administration guidelines are respected. Since both MTX and LV use the membrane transport system, it was hypothesized that selective rescue occurred because transport-deficient, MTX-resistant tumor cells also transported LV poorly. The unsatisfactory clinical results frequently obtained with HDMTX regimens suggest a need to re-evaluate this underlying rationale, especially in view of recent findings concerning the mechanisms of MTX resistance and LV rescue. Experimentally, cells resistant to MTX because of an increased or altered DHFR, decreased metabolism to polyglutamates, or decreased thymidylate synthase activity are not always significantly more sensitive to higher concentrations of MTX. Furthermore, recent studies on human small cell lung cancer cell lines suggest that decreased MTX polyglutamate metabolism and thymidylate synthase activity might be prevalent MTX-resistant mechanisms in human tumors. Selective LV rescue could also occur through mechanisms other than selective uptake by normal tissues. These could include increased activities of the LV activating enzymes (5,10-methenyltetrahydrofolate synthetase and methionine synthase) in normal compared to tumorous tissues and decreased reactivation of DHFR by reduced folates in tumor cells that metabolize MTX more efficiently to polyglutamate derivatives. Ideally, HDMTX regimens should lead to MTX plasma concentrations of 10^{-4} to 10^{-5} *M* for at least 24 hours to overcome MTX transport defects and optimize MTX polyglutamate (MTXPG) formation. LV rescue should be given at the lowest dose and as late as possible after MTX administration to prevent folate build-up in tumor cells and optimize conditions for selective LV rescue. Better knowledge of the mechanisms underlying MTX resistance and LV selectivity could lead to a more rational use of HDMTX regimens.—NCI Monogr 5:61-65, 1987.

MTX plays an important role in the treatment of many cancers, including acute lymphocytic leukemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, head and neck and breast cancer (*1*). Unfortunately, the successful use of MTX has been hampered by de novo or acquired drug resistance. In recent years, HDMTX followed by the reduced folate, 5-formyltetrahydrofolate (LV, folinic acid, citrovorum factor) has been used clinically in the hope that the high plasma levels of MTX achieved would overcome MTX resistance (*2,3*). To be successful, HDMTX therapy must bypass the mechanisms of MTX resistance and LV must selectively rescue normal cells more effectively than tumor cells. We will review the concepts of MTX resistance mechanisms and LV rescue and reexamine the rationale of HDMTX therapy.

MECHANISMS OF MTX RESISTANCE

Decreased Membrane Transport

HDMTX regimens were initially designed to overcome MTX resistance due to defective transport of the drug (*2*). This was felt to be the most important drug resistance mechanism based on in vitro data from human leukemic cells in which the cellular uptake of MTX at lower concentrations (2×10^{-7} *M*) was found to vary more than 5-fold among the different patients tested and to correlate with clinical response, i.e., lower uptake levels being associated with clinical resistance (*4*). Furthermore, the authors identified 2 different MTX transport mechanisms: a temperature-sensitive, saturable and concentrative mode at 2×10^{-7} MTX and a separate temperature-insensitive, nonsaturable and nonconcentrative mode at higher (2×10^{-5} *M*) drug concentrations. These observations were later confirmed by other investigators who also determined that MTX diffusion across the cell membrane at higher concentrations was able to overcome a defect in the concentrative MTX transport system observed at lower drug concentrations (*5,6*).

A number of other MTX resistance mechanisms have been identified experimentally, however, and these are not consistently overcome at higher MTX concentrations.

Increased Levels of DHFR

Large increases in DHFR activity associated with MTX resistance can be produced experimentally in tumor cells by long-term exposure to stepwise increasing concentrations of MTX (*7,8*). Such cells contain increased copies of mRNA and increased numbers of genes coding for DHFR (*8,9*). These experimental findings seem to be clinically relevant, as 1 human small cell lung cancer cell line (*10*),

ABBREVIATIONS: DHFR = dihydrofolate reductase; HDMTX = high-dose methotrexate; K_i = inhibitory constant; LV = leucovorin; MTX = methotrexate; MTXPG = methotrexate polyglutamate.

---

[1] Institut du Cancer de Montréal et Hopital Notre-Dame, Québec, Canada.

[*] *Reprint requests to:* Jacques Jolivet, M.D., Institut du Cancer de Montréal, 1560 Sherbrooke Street East, Montréal, Quebec H2L 4M1, Canada.

and at least 3 other examples of human tumors have been found to have MTX resistance related to gene-amplified increased DHFR levels (*11–13*). The prevalence of this abnormality in clinical MTX resistance is still unknown however. Theoretically, the high concentrations of MTX achieved during HDMTX should be able to bind all the active sites of at least moderately increased DHFR levels and reverse MTX resistance. It is well established clinically and experimentally, however, that MTX exposure leads to an acute increase in its intracellular target enzyme by as yet uncertain mechanisms (*14*). A similar increase in DHFR occurs in cells with genetically amplified levels of the enzyme (*15,16*). This further rapid increase can result experimentally in a failure to saturate and inhibit gene-amplified DHFR (*16*).

Altered Affinity of DHFR

Experimentally, tumor cells resistant to MTX may contain a form of DHFR that has decreased binding affinity for MTX (*17,18*). Some of these enzymes have such low affinity for MTX [MTX inhibitory constant (K_i)$=2.5\times 10^{-4}$ *M;* (*18*)] that plasma MTX concentrations achieved during high-dose therapy would be too low to inhibit them. Recently, clinical examples of MTX-insensitive DHFR have been identified in the blast cells of 3 patients with acute myelogenous leukemia, a disease in which MTX has little clinical antitumor activity (*19*). In 1 of these patients, DHFR activity in vitro was still 40% of control in the face of 10^{-5} *M* MTX, suggesting again that high doses of MTX would not necessarily overcome this mechanism of MTX resistance.

Decreased Metabolism to MTXPGs

The dose- and time-dependent metabolism of MTX to MTXPGs is probably essential to the drug's antitumor activity (*20*). It leads to increased intracellular drug accumulation, tighter drug binding to DHFR, prolonged intracellular drug retention after removal of extracellular drug, and the inhibition of folate-dependent enzymes in addition to DHFR (*20*). Experimentally, the relationship between MTX sensitivity and MTXPG formation has only been examined in a few cell lines, but a general statement can be made that the capacity to produce MTXPGs is associated with drug sensitivity in murine tumors (*21*) and in human leukemic and breast cancer cell lines (*22,23*). Cytotoxicity and MTX metabolism in these lines were tested using brief periods of MTX exposure (24 hr or less), conditions which approximate clinical chemotherapy exposure. Under those circumstances, the prolonged retention of longer chain-length MTXPGs in amounts sufficient to saturate DHFR is the critical characteristic conferred by MTXPG formation and correlates with MTX cytotoxicity. We have recently described a highly resistant human breast cancer cell line with multiple defects including a transport defect, decreased thymidylate synthase activity and profoundly impaired MTXPG formation (*24*). This cell line was still resistant to MTX at high drug concentrations when the transport defect was overcome by diffusion of drug across the cell membrane but the impairment of MTX metabolism persisted. Furthermore, recent data suggest that impaired MTXPG formation might be a prevalent clinical MTX resistance mechanism. In 8 patient-derived cell lines of small cell lung cancer, 4 of 6 lines that were relatively resistant to MTX had as their only defect decreased metabolism to MTXPGs (*25*). In 3 of those lines, enough MTXPGs were formed at higher MTX concentrations to overcome drug resistance. Thus, the higher plasma MTX concentrations achieved during HDMTX therapy could possibly but not always overcome MTX resistance due to deficient MTXPG formation.

Decreased Thymidylate Synthase Activity

Experimentally, tumor cells with lower thymidylate synthase activity are less susceptible to MTX since they produce less dihydrofolic acid and, consequently, they are less dependent on DHFR to recycle the inactive oxidized folates back to active, fully reduced tetrahydrofolates (*26*). This MTX resistance mechanism might also have clinical pertinence since 2 of 6 recently studied small cell lung cancer cell lines were found to have, as their main defect, a 4- to 5-fold lower thymidylate synthase activity than the other cell lines examined (*25*). Furthermore, MTX resistance in these lines was not fully reversed even at 10^{-5} *M* MTX, suggesting again that this MTX resistance mechanism would not necessarily be overcome by HDMTX therapy.

LV RESCUE

The high doses of MTX needed to achieve high plasma drug concentrations have to be administered with the reduced folate antidote, LV, to prevent increased toxicity to normal tissues. The pioneering work of Goldin initially established, in the L1210 murine leukemia model, that high doses of aminopterin or MTX could be administered safely when given in association with LV (*27*). Precise scheduling of folate and antifolate administration was crucial, however, and LV had to be administered 12 to 24 hours after the antifolate in order to reduce the antifolate's toxicity without impairing its antileukemic effectiveness (*27*). Later experiments by Sirotnak et al., using the same experimental model, established in greater detail that there was a narrow window during which LV had to be administered (around 15 to 20 hr after MTX administration) for LV rescue to be effective (*28*). In addition, the same authors determined that the LV dose was also critical per se and that, at higher doses, LV antagonized MTX's antitumor efficacy even when given on an optimal schedule. Consequently, MTX and LV can be used in combination in experimental tumor systems to increase the antifolate's therapeutic index but only within specific LV doses and administration schedules.

Another important feature of LV rescue is its competitive nature. Experimentally, thymidine and a purine, at sufficient concentrations, noncompetitively rescue cells from MTX since they are similarly effective against a wide range of MTX concentrations (*29*). Increasing LV concentrations have to be used for rescue as MTX concentrations are raised, however, revealing a competitive interaction. Furthermore, LV is unable to rescue cells from high MTX concentrations (*29*).

Possible explanations for these observations can be obtained from the known cellular mechanisms of LV rescue. These include: a) membrane interactions; b) replenishment of reduced folates; and c) inhibition of MTXPG formation.

Membrane Interactions

Both LV and MTX use the same active membrane transport system to enter cells. LV inhibits MTX uptake with a K_i of 8.3 μM (*30*) and, in human fibroblasts, 10 μM LV completely prevents the uptake of 1 μM MTX (*31*). Furthermore, if the extracellular reduced folate concentration is sufficiently high, there will be a net displacement of MTX from within cells by a "counterflow" effect through the membrane transport system (*32*). Consequently, if LV is given at high enough concentrations and/or too soon after MTX administration, it can prevent MTX uptake and cytotoxicity. These membrane interactions were originally used to explain "normal-tissue-selective" LV rescue. It was hypothesized that tumor cells were not rescued because insufficient folate entered transport-deficient MTX-resistant tumor cells since both LV and MTX used the same abnormal membrane transport system (*2*).

Replenishment of Reduced Folates

The commonly invoked mechanism to explain LV rescue is that the reduced folate replenishes intracellular folate pools which were depleted by MTX's inhibition of DHFR. LV and its metabolite, 5-methyltetrahydrofolate, first have to be converted to other folates through specific enzymes before replenishing the reduced folate pools. LV is the specific substrate for 5-10 methenyltetrahydrofolate synthetase (EC 6.3.3.2), an enzyme which irreversibly converts LV to 5,10-methenyltetrahydrofolate, a folate that is then readily interconverted to other reduced folates (*33*). 5-Methyltetrahydrofolate, on the other hand, must first yield its methyl group during methionine synthesis and be converted to tetrahydrofolate before replenishing the reduced folate pools (*34*). In situations where MTX resistance is caused by mechanisms other than transport deficiency, however, selective rescue could theoretically occur secondarily to lower activities of methenyltetrahydrofolate synthetase and/or methionine synthase in tumor compared to normal cells. This possibility has already been preliminarily investigated for methionine synthase with conflicting results. While some authors have described tumor cells with lower enzyme activities that were rescued less efficiently with 5-methyltetrahydrofolate than certain normal cells (*35*), others have failed to confirm this observation (*36*) and the hypothesis still awaits confirmation.

The traditional folate repletion model has to be reexamined in view of the known competitive nature of LV rescue, however. If depletion of reduced folates is solely responsible for MTX's mechanism of action, LV rescue should be noncompetitive, i.e., a sufficient LV exposure should replete folate pools and rescue cells even at high MTX concentrations. Recent data suggest possible explanations for the discrepancy between the repletion theory and competitive LV rescue: Allegra et al. have shown that MTX does not significantly deplete intracellular reduced folate pools in human breast cancer cells in culture (*37*). They also show that the increase in dihydrofolate pools observed after DHFR inhibition is self-limited, probably because dihydrofolate polyglutamates and MTXPGs can directly inhibit thymidylate synthetase (*20*). Furthermore, these metabolites can also directly inhibit de novo purine synthesis. Thus, inhibition of thymidylate and purine synthesis by MTX is probably not the result of reduced folate depletion but of direct synthesis inhibition by dihydrofolate polyglutamates and MTXPGs. The high intracellular reduced folate levels achieved following LV exposure must directly compete with these inhibitors at the levels of de novo thymidylate and purine synthesis, thus providing a possible explanation for competitive rescue. Another consequence of increased folate pools in MTX-treated cells is the direct reactivation of DHFR. Reduced folates can compete MTX but not MTXPGs off DHFR (*38*). This phenomenon could help explain both competitive and selective LV rescue. Increasing MTX concentrations will lead to more MTXPG formation and less effective rescue, while rescue will be more effective in normal than in tumor cells since the former are thought to synthesize less MTXPGs (*20*).

Inhibition of MTXPG Formation

More recently, the effects of LV on MTXPG formation and their role in LV rescue has been examined in view of the metabolites' central role in MTX activity. The effect of LV on MTX metabolism depends on the sequence of cellular exposure to LV and MTX. When both compounds are administered simultaneously, LV prevents MTX uptake and consequent MTXPG formation (*30*). When cells are exposed to LV after MTX exposure, the folate can still inhibit, albeit less dramatically, MTXPG synthesis (*39*). This is thought to occur through competition at the level of folylpolyglutamate synthetase, the enzyme responsible for the polyglutamylation of both folates and MTX, since LV is a better substrate than MTX for the mammalian enzyme (*40*). Since the state of folate repletion is an important regulator of MTXPG synthesis (*41*), we have recently examined, in MCF-7 human breast cancer cells, the consequences of LV exposure prior to MTX administration (*42*). This would be analogous to the clinical situation in which repeated HDMTX-LV schedules are administered with MTX exposure of cells previously exposed to high-dose reduced folates. We have shown that LV exposure increases intracellular folate pools and that MTXPG formation is markedly inhibited during subsequent exposure to MTX. Consequently, LV-pretreated cells become significantly less sensitive to MTX than cells grown in regular medium containing folic acid. Thus, repeated LV exposure can produce refractoriness to subsequent doses of MTX.

If LV rescues normal and tumor cells differently, the folate could also possibly have a varying rescue potential among different tumors. This was recently confirmed by Browman et al. who showed that lymphoblasts of T-cell origin required 16- to 46-fold more LV to obtain the same degree of protection from MTX as lymphoblasts of B-cell origin (*43*). The mechanisms underlying this phenomenon are still unclear.

CONCLUSIONS

The often unsatisfactory clinical results obtained with HDMTX therapy suggest that the initial underlying rationale needs to be reevaluated in view of more recent findings concerning the mechanisms of MTX resistance and LV rescue. It is now clear that MTX resistance can occur through a variety of mechanisms which are not consistently overcome by high concentrations of MTX. In addition, it hasn't been clearly shown that LV rescue is indeed selective. The excessive use of LV could have potentially deleterious effects by either rescuing tumors at varying degrees or making them refractory to subsequent MTX therapy.

Based on the above considerations, an "optimal" HDMTX-LV protocol would contain:

1) a sufficient MTX dose schedule to ensure 10^{-4} to 10^{-5} *M* MTX plasma levels for approximately 24 hours. This would ensure adequate drug levels to overcome MTX transport defects and possibly MTXPG formation deficiencies. The length of exposure would optimize MTXPG formation.

2) LV rescue at the lowest dose and as late as possible after MTX therapy. This would prevent extensive folate build up in tumor cells and optimize conditions for selective LV rescue.

Clinical protocols incorporating the above principles have been experimented but deserve more widespread testing (*44*). To use HDMTX more effectively, investigators will eventually have to develop probes to determine in patient tumor samples the expression of key proteins and enzymes involved in MTX resistance and LV rescue with the ultimate goal of identifying a priori tumors likely to respond to this treatment strategy.

REFERENCES

(*1*) Jolivet J, Cowan KH, Curt GA, et al: The pharmacology and clinical use of methotrexate. N Engl J Med 309: 1094–1104, 1983.

(*2*) Frei E III, Jaffe N, Tattersall MH, et al: New approaches to cancer chemotherapy with methotrexate. N Engl J Med 292:846–851, 1975.

(*3*) Frei E III, Blum RH, Pitman SW, et al: High dose methotrexate with leucovorin rescue. Rationale and spectrum of antitumor activity. Am J Med 68:370–376, 1980.

(*4*) Kessel D, Hall TC, Roberts D: Modes of uptake of methotrexate by normal and human leukocytes in vitro and their relation to drug response. Cancer Res 28:564–570, 1968.

(*5*) Warren RD, Nichols AP, Bender RA: Membrane transport of methotrexate in human lymphoblastoid cells. Cancer Res 38:668–671, 1978.

(*6*) Hill BT, Dedhar S, Goldie JH: Evidence that at "high" extracellular methotrexate concentrations the transport barrier is unlikely to be an important mechanism of drug resistance. Biochem Pharmacol 31:263–266, 1982.

(*7*) Biedler JL, Spengler BA: Metaphase chromosome anomaly: association with drug resistance and cell specific products. Science 191:185–187, 1976.

(*8*) Alt FW, Kellems RT, Schimke RT: Synthesis and degradation of folate reductase in sensitive and methotrexate-resistant lines of S-180 cells. J Biol Chem 251:3075–3080, 1976.

(*9*) Kaufman RJ, Brown PC, Schimke RT: Amplified dihydrofolate reductase genes in unstably methotrexate-resistant cells are associated with double minute chromosomes. Proc Natl Acad Sci USA 76:5669–5673, 1979.

(*10*) Curt GA, Carney DN, Cowan KH, et al: Unstable methotrexate resistance in human small-cell carcinoma associated with double minute chromosomes. N Engl J Med 308:199–202, 1983.

(*11*) Horns RC, Dower WJ, Schimke RT: Gene amplification in a leukemic patient treated with methotrexate. J Clin Oncol 2:2–7, 1984.

(*12*) Trent JM, Buick RN, Olson S, et al: Cytologic evidence for gene amplification in methotrexate-resistant cells obtained from a patient with ovarian adenocarcinoma. J Clin Oncol 2:8–15, 1984.

(*13*) Carman MD, Schornagel JH, Rivest RS, et al: Resistance to methotrexate due to gene amplification in a patient with acute leukemia. J Clin Oncol 2:16–20, 1984.

(*14*) Bertino JR, Cashmore A, Fink M, et al: The "induction" of leucocyte and erythrocyte dihydrofolate reductase by methotrexate. II. Clin Pharmacol Ther 6:763–770, 1965.

(*15*) Domin BA, Grill SP, Bastow KF, et al: Effect of methotrexate on dihydrofolate reductase activity in methotrexate-resistant human KB cells. Mol Pharmacol 21: 478–482, 1982.

(*16*) Cowan KH, Goldsmith ME, Ricciardone M, et al: Regulation of dihydrofolate reductase in human breast cancer cells and in mutant hamster cells transfected with a human dihydrofolate reductase minigene. Mol Pharmacol 30: 69–76, 1986.

(*17*) Haber DA, Beverly SM, Kiely ML, et al: Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. J Biol Chem 256: 9501–9510, 1981.

(*18*) Dedhar S, Freisheim JH, Hynes JB, et al: Inhibition of a methotrexate-insensitive dihydrofolate reductase from L5178Y cells by substituted triazines and quinazolines. Biochem Pharmacol 32:922–924, 1983.

(*19*) Dedhar S, Hartley D, Fitz-Gibbons D, et al: Heterogeneity in the specific activity and methotrexate sensitivity of dihydrofolate reductase from blast cells of acute myelogenous leukemia patients. J Clin Oncol 3:1545–1552, 1985.

(*20*) Chabner BA, Allegra CJ, Curt GA, et al: Polyglutamation of methotrexate. Is methotrexate a prodrug? J Clin Invest 76:907–912, 1985.

(*21*) Fabre I, Fabre G, Goldman ID: Polyglutamylation, an important element in methotrexate cytotoxicity and selectivity in tumor versus murine granulocyte progenitor cells in vitro. Cancer Res 44:3190–3195, 1984.

(*22*) McGuire JJ, Mini E, Hsieh P, et al: Role of methotrexate polyglutamates in methotrexate and sequential methotrexate-5-fluorouracil-mediated cell kill. Cancer Res 45:6395–6400, 1985.

(*23*) Jolivet J, Schilsky RL, Bailey BD, et al: Synthesis, retention and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. J Clin Invest 70:351–360, 1982.

(*24*) Cowan KH, Jolivet J: A methotrexate-resistant human breast cancer cell line with multiple defects, including diminished formation of methotrexate polyglutamates. J Biol Chem 259:10793–10800, 1984.

(*25*) Curt GA, Jolivet J, Carney DN, et al: Determinants of the sensitivity of human small-cell lung cancer cell lines to methotrexate. J Clin Invest 76:1323–1329, 1985.

(*26*) Moran RG, Mulkins M, Heidelberger C: Role of thymidylate synthetase activity in development of methotrexate cytotoxicity. Proc Natl Acad Sci USA 76:5924–5928, 1979.

(*27*) Goldin A: Studies with high-dose methotrexate. Historical background. Cancer Treat Rep 62:307–312, 1978.

(*28*) Sirotnak FM, Moccio DM, Dorick DM: Optimization of high-dose methotrexate with leucovorin rescue therapy in the L1210 leukemia and sarcoma 180 murine tumor models. Cancer Res 38:345–353, 1978.

(*29*) Pinedo HM, Zaharko DS, Bull JM, et al: The reversal of methotrexate cytotoxicity to mouse bone marrow cells by leucovorin and nucleosides. Cancer Res 36:4418–4424, 1978.

(*30*) Niethammer D, Jackson RC. *In* Chemistry and Biology of Pteridines (Pfeiderer W, ed). Berlin: De Cruyter, 1976, pp 197–207.

(*31*) Rosenblatt DS, Whitehead VM, Vuchich M-J, et al: Inhibition of methotrexate polyglutamate accumulation in cultured human cells. Mol Pharmacol 19:87–97, 1981.

(*32*) WHITE JC, BAILEY BD, GOLDMAN ID: Lack of stereospecificity at carbon 6 of methyltetrahydrofolate transport in Ehrlich ascites tumor cells. J Biol Chem 253:242–245, 1978.

(*33*) HOPKINS S, SCHIRCH V: 5,10-methenyltetrahydrofolate synthetase. Purifications and properties of the enzyme from rabbit liver. J Biol Chem 259:5618–5622, 1984.

(*34*) DUDMAN NP, SLOWIACZEK P, TATTERSALL MHN: Methotrexate rescue by 5-methyltetrahydrofolate or 5-formyltetrahydrofolate in lymphoblast cell lines. Cancer Res 42:502–507, 1982.

(*35*) HALPERN RM, HALPERN BC, CLARK BR, et al: New approach to antifolate treatment of certain cancers as demonstrated in tissue culture. Proc Natl Acad Sci USA 72:4018–4022, 1975.

(*36*) GROFF JP, BLAKLEY RL: Rescue of human lymphoid cells from the effects of methotrexate in vitro. Cancer Res 38:3847–3853, 1978.

(*37*) ALLEGRA CJ, FINE RL, DRAKE JC, et al: The effect of methotrexate on intracellular folate pools in human MCF-7 breast cancer cells. J Biol Chem 261:6478–6485, 1986.

(*38*) MATHERLY LH, BARLOWE CK, GOLDMAN ID: Antifolate polyglutamylation and competitive drug displacement at dihydrofolate reductase as important elements in leucovorin rescue in L1210 cells. Cancer Res 46:588–593, 1986.

(*39*) GALIVAN J, NIMEC Z: Effects of folinic acid on hepatoma cells containing methotrexate polyglutamates. Cancer Res 43:551–555, 1983.

(*40*) SCHOO MM, PRISTUPA ZB, VICKERS PJ, et al: Folate analogues as substrates of mammalian folypolyglutamate synthetase. Cancer Res 45:3034–3041, 1985.

(*41*) NIMEC Z, GALIVAN J: Characteristics of methotrexate polyglutamate formation in cultured hepatic cells. Arch Biochem Biophys 226:671–680, 1983.

(*42*) JOLIVET J, FAUCHER F, PINARD M-F. Influence of intracellular folates on methotrexate metabolism and cytotoxicity. Biochem Pharmacol. In press.

(*43*) BROWMAN GP, BOOKER L, SPIEGL P: Differential calcium leucovorin protection of human lymphoid cell lines from methotrexate. Cancer Chemother Pharmacol 15:105–110, 1985.

(*44*) STOLLER RG, KAPLAN HG, CUMMINGS FJ, et al: A clinical and pharmacological study of high-dose methotrexate with minimal leucovorin rescue. Cancer Res 39:908–912, 1979.

High-Dose Methotrexate in Osteosarcoma—Mayo Clinic Studies[1]

John H. Edmonson[2,*]

ABSTRACT—While high-dose methotrexate (HDMTX) is active against some osteosarcomas, its efficacy as a single agent in the treatment of metastatic disease has not been uniformly demonstrated. Furthermore, the results of a limited randomized study comparing HDMTX-vincristine with observation alone as primary adjuvant treatment do not support the thesis that HDMTX has been an essential factor in the recently improved control of primary osteosarcoma. Despite this uncertainty concerning the specific contribution of methotrexate to these results, multidrug regimens containing this agent do appear to improve the prognosis of patients with primary osteosarcoma. Regimens which exclude HDMTX reasonably may be studied in the adjuvant treatment of primary osteosarcoma.—NCI Monogr 5:67–69, 1987.

Following the report in 1972 of objective regression of pulmonary metastases from osteosarcoma by Jaffe (*1*), HDMTX has been used extensively in the treatment of this disease. Although clearly an active agent against osteosarcoma, its presumed excellence as a single agent in the treatment of metastatic disease has not been uniformly demonstrated (table 1) (*3–9*).

After questions were raised by Pratt et al. (*5*) in 1979 concerning the relative effectiveness of HDMTX as a single agent in advanced osteosarcomas and concerning the value of escalating doses in methotrexate-resistant cases, other phase II trials of HDMTX in metastatic osteosarcoma have been done. One of these published studies, which was done at the Mayo Clinic (*7*), has been described in further detail (table 2). Among 16 consecutive patients with metastatic osteosarcoma treated with HDMTX and vincristine every 3 weeks, we observed only 1 partial tumor regression of pulmonary nodules. Thirteen of these patients had received no previous cytotoxic drug treatment and 1 other had received adjuvant HDMTX. After failing HDMTX, 4 of the 14 patients who had not received it earlier were then given cyclophosphamide, doxorubicin, and cisplatin (CAP) with partial tumor regression in 2 of the 4. Furthermore, of the 2 patients who failed HDMTX as secondary treatment for metastatic disease no longer sensitive to CAP, 1 had previously achieved complete tumor regression on CAP treatment. Thus, we have not been very favorably impressed by our experience using HDMTX in unresectable metastatic osteosarcoma.

Almost simultaneously with the above metastatic disease trial (1976–1980), we also performed a randomized pilot study of HDMTX as postsurgical adjuvant treatment for patients with primary osteosarcoma (table 3) (*10*). This study, like our phase II metastatic disease study, utilized a vincristine-HDMTX-leucovorin regimen (fig. 1) based on that reported by Jaffe et al. in 1974 (*11*). Our patients, randomized to receive HDMTX, received this, if tolerated, every 3 weeks for 1 year beginning within 1 month after removal of their primary tumors. Patients whose osteosarcomas progressed despite HDMTX received surgical excision of their metastases, if possible, and other chemotherapy with cyclophosphamide and doxorubicin. Control patients, like those randomized to HDMTX, were reevaluated every 6 weeks during the initial year; and if they experienced disease progression they were offered HDMTX or cyclophosphamide and doxorubicin after excision of their metastases.

TABLE 1.—Effects of HDMTX in metastatic osteosarcoma

Authors (Reference)	Individual doses, g/m^2	No. of patients	Objective regression	%	Comments
Jaffe (*1*)	~1.5–15[a] (50–500 mg/kg) q 2–3 weeks	10	2 CR; 2 PR	40	Lung and bone metastases
Rosen et al. (*3*)	8–12 weekly	4	3 CR	75	Lung metastases
Ambinder et al. (*4*)	~0.75–7.5[a] (25–250 mg/kg) q 1–4 weeks	7	0	0	
Pratt et al. (*5*)	2.5–5 weekly	12	1 CR; 2 PR	25	Lung metastases
Kimura (*6*)	~3–9[a] (100–300 mg/kg) weekly	26	3 excellent 2 good	19	Lung metastases
Edmonson et al. (*7*)	3–7.5 q 3 weeks	16	1 PR	6	Vincristine, prior to HDMTX, lung metastases
Vaughn et al. (*8*)	5–12 q 2 weeks	11	0	0	Vincristine, prior to HDMTX
Goorin and Abelson [unpublished data, cited in (*9*)]	12	8	2 PR	25	Lung metastases

[a] Assuming average conversion factor (km) of 30 for converting mg/kg to mg/m^2 (*2*).

ABBREVIATIONS: CAP = cyclophosphamide, doxorubicin, and cisplatin; HDMTX = high-dose methotrexate.

[1] Supported in part by grants CM-57044 and CA-15083 from the National Cancer Institute.

[2] Division of Medical Oncology, Mayo Clinic, Rochester, MN.

[*] *Reprint requests to:* John H. Edmonson, M.D., Division of Medical Oncology, Mayo Clinic, Rochester, MN 55905.

TABLE 2.—Patient characteristics and treatment results

Age, yr	Sex	Histologic type and site of tumor origin	Previous chemotherapy[a]	Highest MTX dose, g/m^2	Best response[b]
43	M	Grade 4 osteosarcoma of spacula (associated with Paget's disease)	None	6	S
16	M	Grade 4 osteoblastic osteosarcoma of femur	None	6	S
19	M	Grade 4 osteoblastic osteosarcoma of tibia	None	6	P
16	M	Grade 4 telangiectatic osteosarcoma of femur	None	6	P
9	M	Grade 4 telangiectatic osteosarcoma of femur	None	7.5	P
51	F	Grade 4 osteosarcoma of vertebra	None	3	S
26	M	Grade 4 fibroblastic osteosarcoma of pelvis	None	7.5	PR for 4 mo
17	M	Grade 4 telangiectatic osteosarcoma of tibia	MeCCNU, CTX-DOX-CDDP	3	P
65	M	Grade 3 osteosarcoma of femur	None	6	P
32	F	Grade 2 osteoblastic osteosarcoma of ethmoid	None	6	S
23	M	Grade 4 small cell osteosarcoma of femur	None	3	P
56	M	Grade 4 osteosarcoma of femur	None	3	S
17	M	Grade 4 chondroblastic osteosarcoma of pelvis	None	6	S
70	M	Grade 4 osteosarcoma of femur	MTX as adjuvant treatment	7.5	S
16	M	Grade 4 osteoblastic osteosarcoma of femur	None	3	P
16	M	Grade 4 osteoblastic osteosarcoma of femur	Pyrazofurin, CTX-DOX-CDDP	3	P

[a] MeCCNU = semustine; CTX = cyclophosphamide; DOX = doxorubicin; and CDDP = cisplatin.
[b] S = stable disease; P = progressive disease; PR = partial regression.

TABLE 3.—Postsurgical adjuvant treatment of primary osteosarcoma: Summary of patient characteristics[a]

Characteristic	Chemotherapy ($N=20$)	No chemotherapy ($N=18$)[b]
Age		
≥21 years	7	9
<21 years	13	9
Sex		
Male	14	10
Female	6	8
Histology		
Osteoblastic + telangiectatic	11	10
Chondroblastic + fibroblastic	9	8
Tumor grade		
2 or 3	5	4
4	15	14
Greatest tumor diameter		
≤9 cm	9	9
>9 cm	11	9
Extremity site		
Proximal to knee or elbow	10	9
Distal to knee or elbow	8	6
Limb girdle	2	3

[a] Reprinted with permission from J Clin Oncol 2:152–156, 1984.
[b] The three nonosteosarcomas are excluded.

As indicated in figure 2, no significant difference in continuous disease-free survival was apparent between the group of 20 patients receiving adjuvant HDMTX and the untreated control group. With median follow-up of approximately 8 years after surgery, our 5-year disease-free survival of approximately 40% is similar to the 44% observed in the original group of 12 patients receiving a similar regimen at the Dana-Farber Cancer Center, most recently updated by Goorin et al. (*12*). Thus, one might presume that this disease-free survival at 5 years represents the real therapeutic efficacy of HDMTX in these 2 small but concordant studies, were it not for the 50% disease-free survival of the concomitant untreated controls included in the Mayo study. Figure 3 demonstrates overall survival for our adjuvant-treated and untreated patients.

Recent reports from limited controlled trials by investigators of the Pediatric Oncology Group (*13*) and by others at the University of California at Los Angeles (*14*) have confirmed that patients treated with multidrug adjuvant chemotherapy regimens containing HDMTX appear to have better prognoses than do patients who receive surgery alone for their osteosarcomas. Because of the number of other agents included, however, the specific importance of HDMTX in this treatment is unclear. The results of our limited controlled study of HDMTX plus vincristine

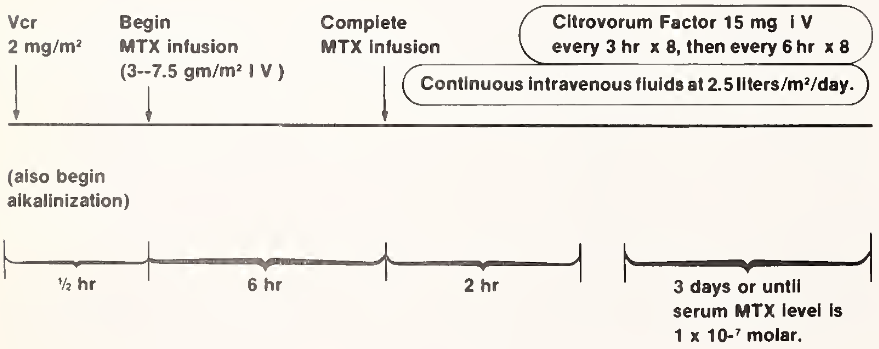


FIGURE 1.—Schema of the HDMTX treatment used in both the metastatic disease and the primary disease studies at Mayo Clinic. MTX = methotrexate; Vcr = vincristine.

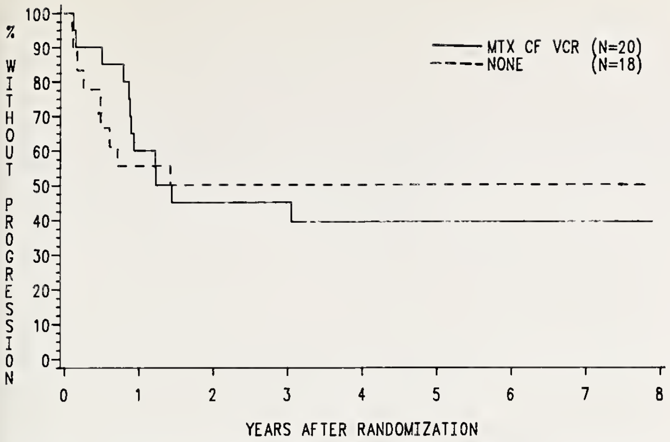


Figure 2.—Estimated probabilities of disease progression following surgery for primary osteosarcoma of bone for 20 patients who received adjuvant HDMTX and for 18 who did not (Kaplan-Meier method) with median follow-up of 8 years after surgery. CF=citrovorum factor.

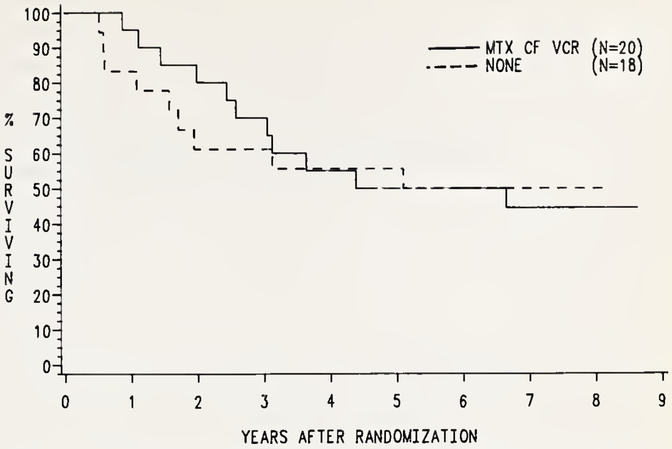


Figure 3.—Estimated probabilities of survival following surgery for primary osteosarcoma of bone for 20 patients who received adjuvant HDMTX and for 18 who did not (Kaplan-Meier method) with median follow-up of 8 years after surgery.

lead us to question whether HDMTX is an essential factor in the improved prognosis observed following this multidrug treatment.

Regimens not containing HDMTX could reasonably be included in future adjuvant chemotherapy studies in patients with primary osteosarcoma.

REFERENCES

(*1*) Jaffe N: Recent advances in the chemotherapy of metastatic osteogenic sarcoma. Cancer 30:1627–1631, 1972.

(*2*) Freireich EJ, Gehan EA, Rall DP, et al: Quantitative comparison of toxicity of anticancer agents in mouse, rat, dog, monkey and man. Cancer Chemother Rep 50:219–244, 1966.

(*3*) Rosen G, Nirenberg A, Juergens H, et al: Response of primary osteogenic sarcoma of single-agent therapy with high-dose methotrexate with citrovorum factor rescue. *In* Current Chemotherapy and Infectious Disease. Proceedings of the 11th International Congress of Chemotherapy and the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy (Nelson JD, Grassi C, eds), vol 2. Washington, DC: The American Society for Microbiology, 1980, pp 1633–1635.

(*4*) Ambinder EP, Perloff M, Ohnuna T, et al: High-dose methotrexate followed by citrovorum factor reversal in patients with advanced cancer. Cancer 43:1177–1182, 1979.

(*5*) Pratt CB, Howarth C, Ransom JL, et al: High-dose methotrexate used alone and in combination for measurable primary or metastatic osteosarcoma. Cancer Treat Rep 64:11–20, 1980.

(*6*) Kimura K: High-dose methotrexate for adult malignancies: Experimental and clinical results. Cancer Bull 33:67–71, 1981.

(*7*) Edmonson JH, Creagan ET, Gilchrist GS: Phase II study of high-dose methotrexate in patients with unresectable metastatic osteosarcoma. Cancer Treat Rep 65:538–539, 1981.

(*8*) Vaughn CB, McKelvey E, Balcerzak SP, et al: High-dose methotrexate with leucovorin rescue plus vincristine in advanced sarcoma: A Southwest Oncology Group study. Cancer Treat Rep 68:409–410, 1984.

(*9*) Malauver MM, Abelson HT, Smit HD: Sarcomas of bone. *In* Cancer, Principles and Practice of Oncology (DeVita VT Jr, Hellman S, Rosenberg SA, eds), 2nd ed. Philadelphia: J.B. Lippincott Co, 1985, p 1318.

(*10*) Edmonson JH, Green SJ, Ivins JC, et al: A controlled pilot study of high-dose methotrexate as postsurgical adjuvant treatment for primary osteosarcoma. J Clin Oncol 2:152–156, 1984.

(*11*) Jaffe N, Frei E III, Traggis D, et al: Adjuvant methotrexate and citrovorum-factor treatment of osteogenic sarcoma. N Engl J Med 291:994–997, 1974.

(*12*) Goorin AM, Frei E III, Abelson HT: Adjuvant chemotherapy for osteosarcoma: A decade of experience. Surg Clin North Am 61:1379–1389, 1981.

(*13*) Link M, Goorin AM, Miser A, et al: The role of adjuvant chemotherapy in the treatment of osteosarcoma of the extremity: Preliminary results of the multi-institutional osteosarcoma study. Proc ASCO 4:237, 1985.

(*14*) Eckardt JJ, Eilber FR, Grant TT, et al: Management of stage IIB osteogenic sarcoma: Experience at the University of California, Los Angeles. Cancer Treat Symp 3:117–130, 1986.

Methotrexate-Leucovorin Factor Rescue Regimens in Diffuse Large Cell Lymphoma

Arthur T. Skarin* and George P. Canellos[1]

ABSTRACT—Early studies of methotrexate (MTX) in non-Hodgkin's lymphoma (NHL) and the rationale for high-dose MTX (HDMTX)-leucovorin (LV) rescue are briefly reviewed. In Phase II studies at the Dana-Farber Cancer Institute, published in 1977, HDMTX-LV (1-7.5 g/m^2 MTX) was given to 12 patients with diffuse large cell lymphoma (DLCL) failing prior chemotherapy. Five (24%) patients responded, including 2 with complete remissions (CR). HDMTX, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone (M-BACOD) was developed (MTX = 3 g/m^2 on day 14 of each cycle) in an effort to reduce relapse between cycles of chemotherapy and also to prevent subsequent CNS relapse. Results in 101 patients published in 1983 showed CR in 72% with a projected survival of 59% out to 5 years. For CR patients, survival was 85% with disease-free survival 74%. In order to render the program more feasible, as well as reduce potential toxicity and cost, moderate-dose MTX-LV (200 mg/m^2 MTX on day 7 and 14) was substituted for HDMTX-LV. Preliminary results from this new protocol, moderate-dose MTX, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone (m-BACOD), as of November 1985 in 80 patients, show CR in 60 (75%) patients. The 2-year survival is 70% with a projected 4-year survival of 60%. Review of both protocols shows similar results including rate of CNS relapse (about 5%). In a multivariate analysis, 3 prognostic groups have been defined, which will allow for new therapeutic strategies in future studies. Prospective randomized trials will be required to determine the role, dose, and schedule of MTX-LV in DLCL.—NCI Monogr 5:71-76, 1987.

Early clinical and pharmacologic studies of daily oral MTX revealed significant activity in patients with advanced NHL, particularly previously treated patients (*1*). Randomization between two dose levels (0.075 vs. 0.15 mg/kg per day) showed a clear dose–response effect with a greater than 2-fold difference in disease regression by a two-fold increase in dose. Of significance, CR, although brief, were only seen in the higher dose schedule. Numerous subsequent publications reported response rates of about 30%.

When MTX was administered as a single agent, toxicity occurred twice as frequently in patients receiving a 2-fold higher dose (*1*). With the availability of LV, a much higher therapeutic ratio was demonstrated with larger doses of MTX. Dose-limiting host toxicity such as oral mucositis, gastrointestinal symptoms, and myelosuppression were no longer a problem. The biochemical, pharmacologic, and clinical rationale for the use of HDMTX with LV rescue in animal and human malignancies has been extensively reviewed (*2*).

In the following sections, the clinical studies involving HDMTX-LV in patients with advanced NHL, mainly with DLCL will be reviewed. The latter term includes patients with the so-called "aggressive" or "poor-prognosis" histologies, mostly comprised of diffuse histiocytic lymphoma (DHL) and its many subtypes as defined immunologically, as well as diffuse undifferentiated non-Burkitt's type lymphoma (DUL).

HIGH-DOSE METHOTREXATE PHASE II STUDIES

Studies of HDMTX-LV were initiated at our institution in July 1975. Results in patients with NHL, mostly previously treated, were published in 1977 (*3*) and updated in 1981 (*4*). MTX doses of 1-7.5 g/m^2 were infused over a 30-minute time interval, followed by LV at 24 hours at a dose of 10 mg/m^2 iv initially, then orally every 6 hours for 12 doses. Of critical importance in this program was generous iv hydration and urinary alkalinization as previously described (*5*) in order to avoid serious renal toxicity. As noted in table 1, 12 patients with diffuse poorly differentiated lymphocytic lymphoma (DPDL) were treated, with 8 responses, including 3 CR. An additional 12 patients with DHL were also treated, and 5 responded. While the responses were quite dramatic, they were generally of short duration, lasting about 2 to 3 months, although 3 patients had prolonged responses of 13, 16, and 21 months. Side effects in the initial study appeared to be independent of dosage and included moderate oral mucositis (3% of courses), gastrointestinal complaints (6% of courses), reversible increase in creatinine (3% of courses), and reversible myelosuppression (4% of courses) (*3*).

ABBREVIATIONS: BACOP = bleomycin, doxorubicin, cyclophosphamide, vincristine, and prednisone; CHOP = cyclophosphamide, doxorubicin, vincristine, and prednisone; COP-BLAM III = cyclophosphamide, vincristine, prednisone, bleomycin, doxorubicin, and procarbazine; CR = complete remission(s); DHL = diffuse histiocytic lymphoma; DLCL = diffuse large cell lymphoma; DPDL = diffuse poorly differentiated lymphocytic lymphoma; DUL = diffuse undifferentiated lymphoma, non-Burkitt's type; HDMTX = high-dose methotrexate; LV = leucovorin; M-BACOD = high-dose methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, and dexamethasone; m-BACOD = moderate-dose methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, and dexamethasone; MTX = methotrexate; NHL = non-Hodgkin's lymphoma; PR = partial remission(s); ProMACE-CytaBOM = prednisone, vincristine, methotrexate, doxorubicin, cyclophosphamide, etoposide, cytosine arabinoside, and bleomycin; ProMACE-MOPP = prednisone, vincristine, methotrexate, doxorubicin, cyclophosphamide, etoposide, mechlorethamine, and procarbazine.

---

[1] Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA.

* *Reprint requests to:* Arthur T. Skarin, M.D., Division of Medical Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

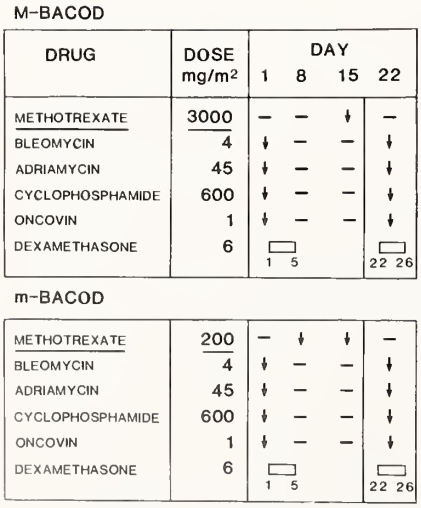
TABLE 1.—Response to HDMTX-LV in non-Hodgkin's lymphoma

Authors (Reference)	MTX dose	LV dose	Histology	CR	PR	NR[a]	Total
Canellos et al. (*4*)	1 to 7.5 g/m^2 30-min infusion	at 24 hr, 10 mg/m^2 iv, orally every 6 hours for 12 doses	DPDL	3	5	4	12
Turman et al. (*6*)	5 to 100 mg/kg 6-hr infusion	at 18 hr, 6 mg im every 6 hours for 3 doses	DPDL	1	1	3	5
Stoller et al. (*7*)	800 to 1350 mg/m^2	at 30 hr, 8 mg/m^2 (median) every 6 hours for 3 doses	DHL	0	1	0	1
Canellos et al. (*4*)	1 to 7.5 g/m^2 30-min infusion	at 24 hr, 10 mg/m^2 iv, orally every 6 hours for 12 doses	DHL	2	3	7	12
Turman et al. (*6*)	5 to 100 mg/kg 6-hr infusion	at 18 hr, 6 mg im every 6 hours for 3 doses	DHL	0	0	1	1
Gomez et al. (*8*)	30 mg/m^2 orally every 6 hours for 4 doses	at 24 hr, 20 mg/m^2 orally every 6 hours for 4 doses	DPDL+DHL	0	6	0	10
Child et al. (*9*)	1 g/m^2 4-hr infusion	at 28 hr, 15 mg orally every 6 hours for 6 doses	Low grade	0	7[b]	2	9
Child et al. (9)	1 g/m^2 4-hr infusion	at 28 hr, 15 mg orally every 6 hours for 6 doses	High grade	0	18[b]	5	23
Cortellaro et al. (*10*)	500 to 7000 mg 6-hr infusion	at 10 hr, 15 mg/m^2 iv every 6 hours for 12 doses	Variable	0	6	3	9
Isacoff et al. (*11*)	50 to 300 mg/kg 4-hr infusion	at 8 hr, 40 mg/m^2 iv then 15 mg every 6 hours for 15 doses	Variable	0	2	0	2

[a] NR = no response.

[b] Response evaluation after 1 dose only.

A summary of several other published studies using HDMTX-LV in NHL is noted in table 1. The doses of MTX and LV vary considerably as do the methods and duration of administration. Results in a total of 84 patients show very few CR, but partial remissions (PR) plus CR occurred in 59% (10 of 17) of DPDL cases, 43% (6 of 14) of DHL cases, 67% (14 of 21) of "variable" and both DPDL and DHL cases, and 78% (25 of 32) of low- and high-grade lymphomas. Two of the reports are from large Phase II studies of HDMTX-LV in solid cancers and contain very few lymphoma patients (*7,11*). One report evaluates only 1 dose of MTX prior to administration of other combined agents (*9*). As with the studies at our institution, responses were generally of short duration (*12*).



M-BACOD

DRUG	DOSE mg/m^2	DAY 1	8	15	22
METHOTREXATE	3000	—	—	↓	—
BLEOMYCIN	4	↓	—	—	↓
ADRIAMYCIN	45	↓	—	—	↓
CYCLOPHOSPHAMIDE	600	↓	—	—	↓
ONCOVIN	1	↓	—	—	↓
DEXAMETHASONE	6	▭ 1 5			▭ 22 26

m-BACOD

DRUG	DOSE mg/m^2	DAY 1	8	15	22
METHOTREXATE	200	—	↓	↓	—
BLEOMYCIN	4	↓	—	—	↓
ADRIAMYCIN	45	↓	—	—	↓
CYCLOPHOSPHAMIDE	600	↓	—	—	↓
ONCOVIN	1	↓	—	—	↓
DEXAMETHASONE	6	▭ 1 5			▭ 22 26

FIGURE 1.—Schemas for the high-dose M-BACOD and moderate-dose m-BACOD protocols. Twenty-four hr after methotrexate, leucovorin rescue is administered, 10 mg/m^2 iv or po q6h×12 for high dose and q6h×8 for moderate dose. The other drugs are given iv, except for dexamethasone, which is oral. The second cycle starts on day 22. A total of 10 cycles are administered.

HIGH-DOSE METHOTREXATE MULTIDRUG REGIMENS

Since HDMTX-LV showed significant activity in patients failing prior standard combination chemotherapy regimens, a new protocol was designed for initially untreated patients. The new protocol, M-BACOD (fig. 1), was based on the rationale noted in table 2. It replaced bleomycin, doxorubicin, cyclophosphamide, vincristine and prednisone (BACOP), which had a relatively low cure rate due to disease regrowth between cycles of therapy, and a rather high CR relapse rate and CNS involvement rate (*13*). In the M-BACOD protocol, HDMTX was administered at a dose of 3 g/m^2 on day 14 with appropriate pretreatment hydra-

TABLE 2.—Rationale for HDMTX-LV in the M-BACOD regimen

1. Activity of low-dose MTX
2. Results of HDMTX
    CR 5 of 22 (23%)
    CR + PR 13 of 22 (59%)
3. HDMTX is non-myelosuppressive
    A. Combine with other agents
    B. Give at WBC nadir
    C. Possibly prevent tumor regrowth between cycles
4. Effective in CNS lymphoma
    A. Potential prevention of CNS relapse

TABLE 3.—Comparison of BACOP and M-BACOD in DHL/DUL[a]

	No.	CR	CR relapse	5-yr survival all patients
BACOP	22	11 (50%)	7 (64%)	28%
		$P=.04$	$P=.02$	$P=.001$
M-BACOD	101	73 (72%)	19 (26%)	58%

[a] Data compiled from Skarin et al. (*12,13*).

tion and urinary alkalinization, followed by LV rescue at 24 hours. Chemotherapeutic agents were administered every 21 days for 10 cycles. Dexamethasone replaced prednisone, since it appeared that the former penetrated the CNS more effectively (to reduce CNS relapse) and had less adverse effect on leukocyte function (to reduce infection). A total of 101 patients with advanced NHL were treated on this regimen from July 1976 to July 1981 (*14*). The results were identical in patients with DHL (82 cases) and DUL (13 cases). Six early deaths occurred, due to sepsis in 3, lung failure in 1, and acute myocardial infarction in 2 patients. The CR rate was higher and more durable than the previous BACOP regimen. Most relapses occurred within the first 2 years. The 2 regimens are compared in table 3. The superiority of M-BACOD is quite evident. Survival of all patients and CR patients is noted in figures 2 and 3, also demonstrating the higher cure rate of the HDMTX-LV regimen. Relapse in the CNS occurred in 4 of 73 (5.4%) CR patients on M-BACOD compared to 10% on BACOP. Because of small numbers and lack of randomization, firm conclusions cannot be made regarding prophylaxis of CNS relapse by HDMTX.

The overall results of M-BACOD were quite impressive and compared favorably to other regimens. However, wide-scale use of the program was limited by several factors, noted in table 4 and, therefore, the program was modified by use of moderate-dose MTX, administered on day 8 and 15 of each 21-day cycle, at a dose of 200 mg/m^2. Preliminary results of m-BACOD were presented in 1983 (*15*) and updated in 1985 (*16*). A CR rate of 75% has been achieved in 80 patients, with median follow-up of CR patients about 3 years. CNS relapse has occurred in 4 of 60 (6.7%) CR patients. Moderate-dose MTX was not used with CNS prophylaxis in mind. The results are identical to the high-dose M-BACOD program. The major toxicities of both programs are compared in table 5. As noted, m-BACOD resulted in more mucositis, possibly related to less rigid criteria for hydration. Increasing the dose and frequency of LV rescue reduces but does not eliminate mucositis. Mucositis may be related to use of moderate-dose MTX on day 8 plus 15 in conjunction with toxicity from day 1 agents. The high rate of leukopenia may also relate to similar pharmacologic factors.

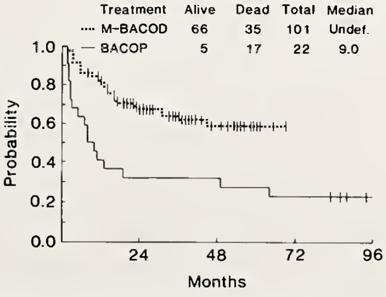


FIGURE 2.—Survival of all patients from onset of therapy with BACOP or M-BACOD (*12,13*).

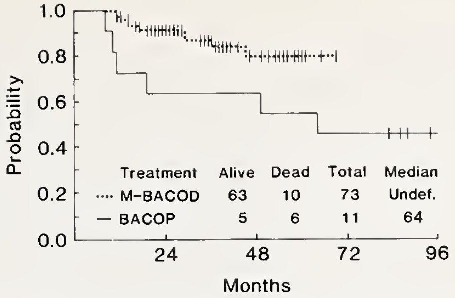


FIGURE 3.—Survival of CR patients from end of therapy with BACOP or M-BACOD (*12,13*).

The results of M-BACOD and m-BACOD are compared to other selected first-, second-, and third-generation regimens that utilize HDMTX-LV rescue in table 6. The percent long-term complete responders, which translates into the cure rate, increases with the newer regimens. However, the median follow-up of the latter programs is limited, and further observation is required before firm conclusions can be made. Also, other non-MTX containing regimens such as cyclophosphamide, vincristine, prednisone, bleomycin, doxorubicin and procarbazine (COP-BLAM-III) (*22*) may be equally effective, especially when certain prognostic categories are compared (see below).

TABLE 4.—Advantages of moderate-dose MTX over high-dose MTX

1. No need for urinary alkalinization and vigorous hydration
2. No need to monitor blood levels
3. Avoid potentially serious renal and other toxicity
4. Reduced cost
5. More wide-scale use

TABLE 5.—Major toxicity related to program[a]

	Moderate-dose m-BACOD (80 patients)	High-dose M-BACOD (101 patients)
Mucositis	32 (40%) 6% of courses	10 (10%) 1% of courses
Increase in creatinine	3 (3.8%)	10 (10%)
Skin rash	1 (2%)	3 (3%)
Acute CNS syndrome	0	1 (1%)
Leukopenia with fever	23 (28%)	8 (8%)
Fatal	2 (2.5%)	3 (3%)
Pulmonary infiltrates related to bleomycin	16 (20%)	12 (12%)
Adriamycin cardiotoxicity	3 (3.8%)	2 (2%)

[a] Data compiled from Skarin et al. (*13,15*). Reprinted with permission from Advances in Cancer Chemotherapy: Update on Treatment of Diffuse Large Cell Lymphoma, 1986, pp. 23–29.

TABLE 6.—MTX-LV-containing regimens in mainly previously untreated DLCL

Author (Reference)	Regimen[a]	MTX dose, mg/m^2	No. of patients	CR, %	Relapse-free survival, %	Long-term CR, %	Median follow-up CR patients yr
Gaynor et al. (*17*)	COMLA	120	42	55	87	48	>5
Todd et al. (*18*)	ACOMLA	120	20	65	85	55	>3
Skarin et al. (*14*)	M-BACOD	3000	95	77	74	57	5
Skarin et al. (*16*)	m-BACOD	200	80	75	77	58	3
Fisher et al. (*19*)	ProMACE-MOPP	1500	79	73	82	60	>4
Fisher et al. (*20*)	ProMACE-CytoBOM	120	28	89	88	78	2
Klimo and Connors (*21*)	MACOP+Bleo	400	61	84	90	75	2

[a] *Abbreviations:* COMLA=cyclophosphamide, vincristine, MTX, LV, and cytosine arabinoside; ACOMLA=doxorubicin, cyclophosphamide, vincristine, MTX, LV, and cytosine arabinoside; Bleo=bleomycin.

TABLE 7.—MTX-LV-containing regimens in mainly previously treated NHL

Author (Reference)	Regimen[a]	Patient features	Histology	CR/Total	Comment
Gomez et al. (*8*)	MTX - COP	No prior chemotherapy	DHL	3/3	Survival 10, 25+, 32+ months
″ ″	MTX-COP	″ ″ ″	DPDL	4/7	2 relapse, 5/7 alive
″ ″	MTX - CHOP	Prior chemotherapy	DHL	3/5	2/5 alive, relapse-free
″ ″	MTX - CHOP	″ ″	DPDL	1/6	all relapse
Child et al. (*9*)	MTX - CHOP	Minimal prior chemotherapy	High grade	13/21 (62%)	only 1 CR relapsed
″ ″	MTX - CHOP	Prior chemotherapy	Low grade	16/25 (64%)	most patients died
Cabanillas, et al. (*23*)	IMVP-16	Prior chemotherapy	Variable (DLCL)	18/52 (37%)	10 CR patients relapse-free
Helms et al. (*24*)	VP-16, BCNU, Bleo, MTX	Prior chemotherapy	DHL	4/15 (25%)	CR duration 4, 12, 24, 32 months

[a] *Abbreviations* as in table 6 plus: COP=cyclophosphamide, vincristine, and prednisone; IMVP-16=ifosfamide, methotrexate, and etoposide; VP-16=etoposide; BCNU=carmustine.

MTX in variable doses has been combined with other agents in the so-called salvage programs designed for patients with NHL failing standard multidrug regimens. Several such programs are summarized in table 7. As noted, the CR rates are fairly respectable, but range from 25% to 62%, depending upon histology and other factors. Total responses (CR+PR) are much higher. The largest experience is from Houston, using the IMVP-16 (ifosfamide, methotrexate, etoposide) regimen (*23*). The latter consists of ifosfamide 1 g/m^2 daily for 5 days, MTX 30 mg/m^2 on days 3 and 10, and VP-16, 100 mg/m^2 daily for 3 days, with repeat courses every 21 days. Of 52 patients with recurrent NHL (mainly DHL), 32 (62%) responded including CR in 18 (37%). Ten of the CR patients continued relapse-free beyond 1 year.

IMPORTANCE OF PROGNOSTIC FACTORS

An awareness of multiple prognostic factors is critically important in the treatment planning of DLCL. While the parameters of response and survival were not very relevant with early treatment programs, they must be considered as an integral part of any randomized study or for selection of the optimal multi-agent program. With many of the treatment programs, factors such as age, sex, stage of disease, histologic subtype, and previous therapy have significantly affected response or survival. More recent studies have emphasized bulk disease, constitutional symptoms, and extent of extra nodal disease as important prognostic variables. Factors found to be significant at the .05 level in univariate analysis may not be found to be significant in a proportional-hazards-model regression analysis (multivariate analysis).

Recently, a critical biostatistical analysis of our M-BACOD and m-BACOD regimens has been carried out (*25*). A total of 127 patients who had complete laboratory, radiologic, pathologic, and clinical data available were reviewed and it was found that the results of both regimens were the same (table 8 and fig. 4B). As a consequence, the data were combined and several prognostic variables were analyzed (*26*). Response was not affected by age, sex,

TABLE 8.—Comparison of high dose (M) and moderate dose (m) BACOD regimens in DHL/DUL

				Survivals		
	No.[a]	CR	PR	1-yr	2-yr	5-yr
M-BACOD	87	63 (72%)	16 (18%)	81%	62%	56%
m-BACOD	40	29 (73%)	5 (15%)	83%	60%	—

[a] No. of patients who had all laboratory, x-ray, pathology, and clinical data available for review (*25*).

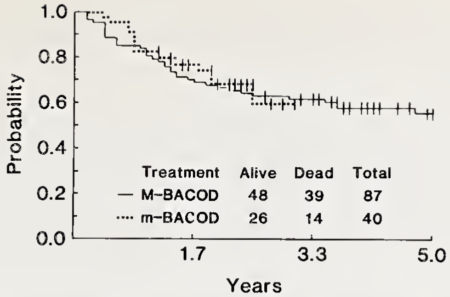


FIGURE 4.—Survival of fully evaluable patients from onset of therapy with M-BACOD or m-BACOD (*25*).

stage, involvement of marrow, liver or gastrointestinal tract, number of extranodal sites, or histologic/immunologic subgroup (B vs. T cell, immunoblastic sarcoma (IBS) vs. other, grade of tumor). At least 6 factors were predictive of response by univariate analysis, but only performance status and tumor mass >10 cm and number of extranodal sites evolved as independent prognostic factors by multivariate analysis. By using these factors, the patients were separated into 3 prognostic groups: a low-risk group (about 1/2 of the patients) with a 70% probability of surviving 5 years; a middle-risk group (about 1/3 of patients) with a 50% probability; and a high-risk group (about 1/6 of patients) with only a 20% chance of surviving 5 years. The latter patients may benefit from new innovative treatment strategies.

SUMMARY

Several studies have shown the increased therapeutic ratio of single agent HDMTX-LV and activity in patients with advanced NHL, particularly aggressive types such as DLCL. High- and moderate-dose MTX has been incorporated into several multidrug regimens resulting in increasing CR rates and long-term survival as the programs become more complex and intensive (1st, 2nd, and 3rd generation protocols). Variable doses of MTX-LV have been combined with other agents in several salvage programs which have been effective in some patients failing standard regimens.

In previously untreated patients with DLCL, prospective randomized trials, however, will be required to determine the role, dose, and schedule of MTX-LV rescue, and the optimal combination of other agents. Such an approach has already been started in at least 3 randomized studies: cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) vs. m-BACOD vs. prednisone, vincristine, MTX, doxorubicin, cyclophosphamide, etoposide, cytosine arabinoside, and bleomycin (ProMACE-CytaBOM) vs. MACOP-bleomycin (Southwest Oncology Group); CHOP vs. m-BACOD (Eastern Cooperative Oncology Group and Cancer and Leukemia Group B): and CHOP vs. prednisone, vincristine, MTX, doxorubicin, cyclophosphamide, etoposide, mechlorethamine, and procarbazine (ProMACE-MOPP) (Bonadonna, Milan, personal communication). Stratification by major prognostic groups as outlined above will be important in interpreting the final results of these studies.

REFERENCES

(*1*) FREI E III, SPURR CL, BRINDLEY CO, et al: Clinical studies of dichloromethotrexate. Clin Pharmacol Ther 6:160–171, 1965.

(*2*) FREI E III, JAFFE N, TATTERSALL MH: New approaches to cancer chemotherapy with methotrexate. N Engl J Med 292:846–851, 1975.

(*3*) SKARIN AT, ZUCKERMAN KS, PITMAN SW, et al: High-dose methotrexate with folinic acid in the treatment of advanced non-Hodgkin lymphoma including CNS involvement. Blood 50:1039–1047, 1977.

(*4*) CANELLOS GP, SKARIN AT, ROSENTHAL DS, et al: Methotrexate as a single agent and in combination chemotherapy for the treatment of non-Hodgkin's lymphoma of unfavorable histology. Cancer Treat Rep 65:125–129, 1981.

(*5*) PITMAN SW, PARKER LM, TATTERSALL MH: Clinical trial of high-dose methotrexate (NSC-740) with citrovorum factor (NSC-3590)—toxicologic and therapeutic observations. Cancer Chemother Rep 6:43–49, 1975.

(*6*) TURMAN S, COLEMAN M, SILVER RT, et al: High dose methotrexate with citrovorum factor in adult resistant lymphoma. Cancer 40:2823–2828, 1977.

(*7*) STOLLER RG, KAPLAN HG, CUMMINGS FJ, et al: A clinical and pharmacological study of high-dose methotrexate with minimal leucovorin rescue. Cancer Res 39:908–912, 1979.

(*8*) GOMEZ GA, STUTZMAN L, MOAYERI H, et al: Combinations of methotrexate (COP or CHOP) in the treatment of previously untreated and treated lymphomas. Cancer Treat Rep 66:43–47, 1982.

(*9*) CHILD JA, BARNARD DL, CARTWRIGHT SC, et al: A pilot study of cyclical chemotherapy with high-dose methotrexate and CHOP (MTX-CHOP) in poor-prognosis non-Hodgkin's lymphoma (NHL). Cancer Chemother Pharmacol 11:153–158, 1983.

(*10*) CORTELLARO M, BOSCHETTI C, DI PADOVA F, et al: Methotrexate with N^5-methyltetrahydrofolic acid in the treatment of non-Hodgkin's lymphomas and acute leukemias. Hematologica 68:74–86, 1983.

(*11*) ISACOFF WH, EILBER F, TABBARAH H, et al: Phase II clinical trial with high-dose methotrexate therapy and citrovorum factor rescue. Cancer Treat Rep 62:1295–1304, 1978.

(*12*) FREI E III, BLUM RH, PITMAN SW, et al: High-dose methotrexate with leucovorin rescue. Am J Med 68:370–376, 1980.

(*13*) SKARIN AT, ROSENTHAL DS, MOLONEY WC, et al: Combination chemotherapy of advanced non-Hodgkin's lymphoma with bleomycin, adriamycin, cyclophosphamide, vincristine and prednisone (BACOP). Blood 49:325–333, 1977.

(*14*) SKARIN AT, CANELLOS GP, ROSENTHAL DS, et al: Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). J Clin Oncol 1:91–98, 1983.

(*15*) SKARIN A, CANELLOS G, ROSENTHAL D, et al: Moderate dose methotrexate (m) combined with bleomycin (B), adriamycin (A), cyclophosphamide (C), oncovin (O) and dexamethasone (D), m-BACOD, in advanced diffuse histiocytic lymphoma (DHL). Proc ASCO 2:220, 1983.

(*16*) SKARIN AT, CANELLOS GP, ROSENTHAL DS, et al: Moderate dose m-BACOD in advanced diffuse large cell lymphoma—an interim report. *In* Advances in Cancer Chemotherapy: Update on Treatment of Diffuse Large Cell Lymphoma (Skarin AT, ed). New York: Projects in Medicine, Park Row, 1986, pp 23–29.

(*17*) GAYNOR ER, ULTMANN JE, GOLOMB HM, et al: Treatment of diffuse histiocytic lymphoma (DHL) with COMLA (cyclophosphamide, oncovin, methotrexate, leucovorin, cytosine arabinoside): A 10-year experience in a single institution. J Clin Oncol 3:1596–1604, 1985.

(*18*) Todd M, Cadman E, Spiro P, et al: A follow-up of a randomized study comparing two chemotherapy treatments for advanced diffuse histiocytic lymphoma. J Clin Oncol 2:986–993, 1984.

(*19*) Fisher RI, DeVita VT, Hubbard SM, et al: Diffuse aggressive lymphomas: increased survival after alternating flexible sequences of ProMACE and MOPP chemotherapy. Ann Intern Med 98:304–309, 1983.

(*20*) Fisher RI, DeVita VT, Hubbard SM, et al: Randomized trial of ProMACE-MOPP vs. ProMACE-CytaBOM in previously untreated, advanced stage, diffuse aggressive lymphomas. Proc ASCO 3:242, 1984.

(*21*) Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. Ann Intern Med 102:596–602, 1985.

(*22*) Coleman M, Boyd DB, Bernhardt B, et al: COPBLAM III: Combination chemotherapy for diffuse large cell lymphoma (LCL) with cyclophosphamide (C), infusional oncovin (O), prednisone (P), infusional bleomycin (BL), adriamycin (A) and matulane (M). Proc ASCO 3:246, 1984.

(*23*) Cabanillas F, Hagemeister FB, Bodey GP, et al: IMVP-16: An effective regimen for patients with lymphoma who have relapsed after initial combination chemotherapy. Blood 60:693–697, 1982.

(*24*) Helms SR, Obalon DJ, Braylan RC, et al: Etoposide, carmustine, bleomycin, and methotrexate with leucovorin rescue as re-treatment for unfavorable non-Hodgkin's lymphoma. Cancer Treat Rep 69:783–786, 1985.

(*25*) Shipp M, Klatt M, Harrington D, et al: M-BACOD and m-BACOD in the treatment of unfavorable prognosis lymphoma: analysis of prognostic variables. Proc ASCO 4:205, 1985.

(*26*) Shipp M, Harrington DP, Klatt MM, et al: Identification of major prognostic subgroups of patients with large-cell lymphoma treated with m-BACOD or M-BACOD. Ann Intern Med 104:757, 1986.

A Randomized Trial of High-Dose Methotrexate Versus Standard-Dose Methotrexate Following Cyclophosphamide, Doxorubicin (Adriamycin), Vincristine, and Prednisone With or Without Bleomycin in the Therapy of Diffuse Large Cell Lymphoma: Preliminary Report of Cancer and Leukemia Group B Study 7851[1]

Sandra J. Ginsberg[2,*] James R. Anderson,[3] Arlan J. Gottlieb,[2] Clara D. Bloomfield,[4] Larry Norton,[5] Maurice Barcos,[6] and James F. Holland[5]

ABSTRACT—Between 1979 and 1985, 166 patients with diffuse large cell (histiocytic) lymphoma were randomized to receive therapy with 3 courses of cyclophosphamide, doxorubicin (Adriamycin), vincristine, and prednisone (CAVP), with or without low-dose bleomycin, by continuous iv infusion. Responders were further randomized to 3 weeks of therapy with either high-dose methotrexate (3 g/m^2 iv weekly with leukovorin rescue) or low-dose methotrexate (30 mg/m^2 orally weekly without rescue). Therapy was concluded with 3 additional courses of CAVP. No significant differences among the 4 treatment programs were observed in complete response rates (ranging from 46% to 51%) or in failure-free survival. Of the 38 relapses that have occurred in patients treated with low-dose methotrexate, 5 included relapse in the central nervous system in conjunction with systemic relapse. However, none of 31 relapses observed in patients receiving high-dose methotrexate have occurred with involvement of the central nervous system. Patients entering this study with "B" symptoms had significantly poorer treatment results than those patients entering study without "B" symptoms.—NCI Monogr 5:77-80, 1987.

Between April 1979 and June 1985, the Cancer and Leukemia Group B (CALGB) studied the treatment of patients with Stage III or IV non-Hodgkin's lymphoma with histologic subtypes of diffuse poorly differentiated lymphocytic, diffuse mixed, nodular large cell (histiocytic), nodular and diffuse large cell and diffuse large cell lymphoma (Rappaport classification). Patients were initially randomized to receive 3 cycles of CAVP given every 3 weeks (*1,2*) with or without low-dose infusion bleomycin. Patients in complete or partial response were then randomly assigned to 3 weekly courses of either low-dose methotrexate or high-dose methotrexate with leucovorin rescue. Methotrexate therapy was followed by 3 additional courses of CAVP. The current report details our preliminary findings in patients with diffuse large cell lymphoma.

PATIENTS AND METHODS

Patient eligibility.—Patients with Stage III or IV non-Hodgkin's lymphoma with histologic subtypes of diffuse poorly differentiated lymphocytic, diffuse mixed, nodular large cell, nodular and diffuse large cell, and diffuse large cell lymphoma were eligible for study. Eligibility criteria included no prior chemotherapy, a creatinine clearance of at least 75 ml/minute, adequate hepatic function, and adequate hematologic reserve unless abnormalities were due to lymphoma. Central review of diagnostic pathologic slides by the National Lymphoma Panel was required. Informed consent for treatment was obtained from all patients.

Chemotherapy program.—Patients were randomized initially to receive 3 cycles of cyclophosphamide (750 mg/m^2 iv day 1), Adriamycin (50 mg/m^2 iv day 1), vincristine (2 mg iv day 1), and prednisone (100 mg per day orally for 5 days) given every 21 days (CAVP), or 3 cycles of CAVP plus bleomycin given as a continuous intravenous infusion of 2 units/day on days 1–5 (CAVPB). Patients were restaged at the end of this initial phase of therapy. Those attaining at least a partial response and having an adequate creatinine clearance were then randomized to receive high-dose methotrexate (3 g/m^2 iv weekly) with citrovorum factor rescue (10 mg/m^2 every 6 hours for 12 doses) or low-dose methotrexate (30 mg/m^2 orally weekly) without rescue for 3 weeks. Those patients who could not receive high-dose methotrexate because of low creatinine clearance received an extra cycle of CAVP and are included in the analysis of outcome of the initial phase of therapy and the entire treatment program, but not in the analysis of the methotrexate phase of the study. Patients with continuing partial or complete responses at the end of the methotrexate phase concluded therapy with 3 cycles of CAVP given every three weeks. Patients were reevaluated after completing the final phase of protocol therapy and then observed for recurrence

---

ABBREVIATIONS: CALGB = cancer and Leukemia Group B; CAVP = cyclophosphamide, doxorubicin (Adriamycin), vincristine, and prednisone; CAVPB = cyclophosphamide, doxorubicin, vincristine, prednisone and bleomycin.

---

[1] Supported by grants CA21060 (SJG and AJG), CA33601 (JRA), CA04457 (JFH and LN), CA16450 (CDB) and CA02599 (MB) from the National Cancer Institute, Department of Health and Human Services and a grant from the T. J. Martell Foundation (JRA).

[2] State University of New York Health Science Center, Syracuse, NY.

[3] Department of Biostatistics, Harvard School of Public Health, Boston, MA.

[4] University of Minnesota School of Medicine, Minneapolis, MN.

[5] Mount Sinai Hospital School of Medicine, New York, NY.

[6] Roswell Park Memorial Institute, Buffalo, NY.

[*] *Reprint requests to:* Sandra J. Ginsberg, M.D., 1301 Trumansburg Rd., Ithaca, NY 14850.

of disease. Patients not achieving a complete remission after the final phase of treatment were "off-protocol" and received additional therapy as deemed appropriate.

RESULTS

Four hundred thirteen patients with Stage III or IV intermediate- or high-grade non-Hodgkin's lymphoma were entered on study. Eight patients were "cancelled" and never began protocol therapy. Pathologic review by the National Lymphoma Panel has been completed for 358 of the remaining 405 patients. One hundred sixty-nine of the 358 patients (46%) have been classified as having diffuse large cell (histiocytic) lymphoma. Three of these 169 patients were ineligible for study, leaving 166 eligible patients with diffuse large cell lymphoma for this analysis. The current data are updated to December 1985. The median follow-up time is 42 months.

The clinical characteristics of the 166 eligible patients are shown in table 1. The patients were elderly: 24% of the patients were at least 70 years of age and more than half the patients were at least 60 years of age. Half the patients were male and approximately half had "B" symptoms. Eighty-four patients were randomized to receive initial therapy with CAVP while 82 were randomized to receive CAVPB. Patients were evenly balanced across initial therapy regimens with regard to age, gender, symptoms, and exposure to prior radiation therapy. Somewhat fewer patients with Stage IV disease were randomized to receive initial therapy with bleomycin.

Toxicity

The toxicities experienced during the initial phase of therapy were those expected from the doses of CAVP administered. They did not differ with the addition of bleomycin. No patient receiving therapy with bleomycin experienced severe or life-threatening pulmonary toxicity. Nine patients were ineligible for the methotrexate randomization because of low creatinine clearance or other medical problems. Six percent of patients who received high-dose methotrexate with rescue had severe renal toxicity; no severe renal toxicity was observed with low-dose methotrexate ($P=0.09$). Twelve percent of patients who received high-dose methotrexate had severe or life threatening myelosuppression as compared to 3% of patients who received low-dose methotrexate ($P=0.14$).

TABLE 1.—Clinical characteristics of 166 patients with diffuse large cell lymphoma

	Phase I therapy		
	CAVP	CAVPB	Total
No. patients	84	82	166
Age, yr			
<50	24%	13%	19%
50–59	19%	26%	22%
60–69	36%	34%	35%
70+	21%	27%	24%
Gender			
Male	49%	53%	51%
Female	51%	47%	49%
Prior therapy			
None	90%	90%	90%
Radiotherapy	10%	10%	10%
Stage/symptoms at treatment			
IIIA	15%	24%	20%
IIIB	11%	16%	13%
IVA	33%	28%	31%
IVB	41%	31%	36%

TABLE 2.—Complete response rates after initial treatment phase, methotrexate phase, and completion of therapy for diffuse large cell lymphoma patients[a]

Initial phase	Methotrexate phase	Conclusion of therapy
CAVP—20%	HMtx—33%	46%
	LMtx—23%	51%
CAVPB—28%	HMtx—37%	46%
	LMtx—34%	51%

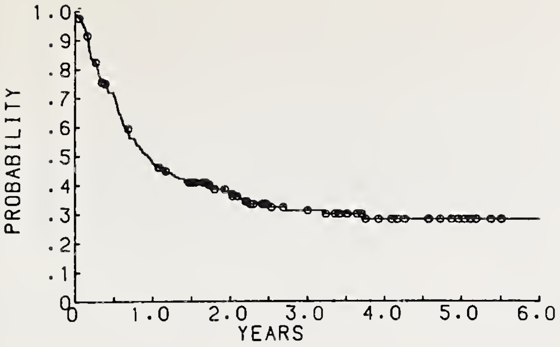
[a] Patients were reevaluated after each phase of therapy. The initial phase of treatment employed 3 courses of therapy with CAVP ± bleomycin. High-dose methotrexate with rescue (HMtx) or low-dose methotrexate (LMtx) were then given weekly for 3 weeks. Three courses of CAVP given every 3 weeks concluded therapy.

Response

The complete response rates to each phase of therapy are shown in table 2. There was no significant difference in complete response frequency between CAVP or CAVPB during the initial phase of therapy. Neither was there a significant difference in the overall complete response rates attained by the 4 therapeutic combinations, with complete response rates ranging from 46% to 51%. Complete response rates were higher for patients less than 70 years of age ($P=0.01$) and for Stage IIIA patients ($P=0.03$).

Time to Treatment Failure

Patients were considered treatment failures if they developed progressive disease, relapsed after response to therapy, or died from any cause. Analysis of time from study entry to treatment failure showed a median time to treatment failure of 10 months for all patients. There were not any significant differences in time to treatment failure according to age ($P=0.82$), stage ($P=0.59$) or type of initial therapy (CAVPB or CAVP) ($P=0.50$, fig. 1). The 3-year failure-free survival of the 84 patients treated with CAVP without bleomycin was estimated to be 28% as compared to 35% for the 82 patients treated with bleomycin. Failure-free survival differed significantly by gender ($P=0.04$, 3-year failure-free survival: male, 28%; female, 36%) and by symptoms regardless of stage ($P=0.003$, 3-year failure-free survival: asymptomatic, 42%; symptomatic, 20%). Analysis of time from entry into the methotrexate phase of therapy to treatment failure showed no statistically significant differences between methotrexate regimens ($P=0.58$, fig. 2). There were also no differences in



STUDY	ALIVE	DEAD	TOTAL	MEDIAN
—— 7851	58	108	166	0.9

FIGURE 1.—Time to treatment failure for all patients from registration.

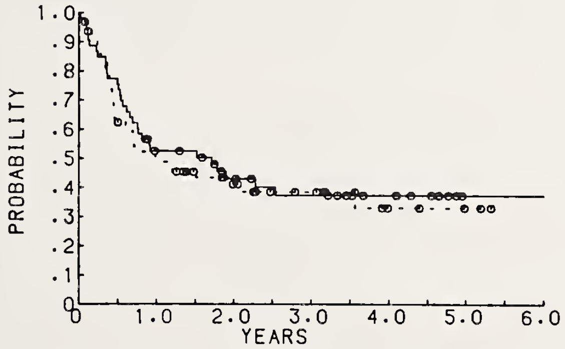
failure-free survival between the various combinations of initial and methotrexate phases of therapy ($P=0.76$, fig. 3).

A total of 69 patients relapsed following response to therapy. Five of the 69 relapses included recurrence in the central nervous system concurrent with systemic relapse. All 5 were patients who had received low-dose MTX ($P=0.06$).

DISCUSSION

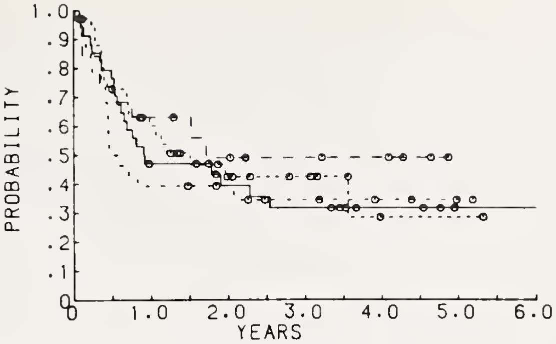
In an effort to improve a standard 4-drug therapy for non-Hodgkin's lymphoma (cyclophosphamide, Adriamycin, vincristine, and prednisone) (*1–3*), 2 additional drugs (methotrexate and bleomycin), both active as single agents, were added in a manner that allowed their individual and collective contribution to be evaluated.

Methotrexate, as a single agent, has shown significant activity in large cell lymphoma (*4*). Increased activity has been observed when the drug was used in higher doses as a single agent and in various combination programs (*4–10*). In addition, high-dose methotrexate given intravenously attains potentially therapeutic levels in the central nervous system (*7–9*).



SUBSEQ RX	ALIVE	DEAD	TOTAL	MEDIAN
—— HI MTX	22	31	53	1.7
- - LOW MTX	26	37	63	1.1

FIGURE 2.—Time to treatment failure (disease progression or death) from initiation of methotrexate therapy for patients with diffuse histiocytic lymphoma. Patients were randomly assigned to 2 groups following initial therapy with CAVP or CAVPB. Hi MTX=methotrexate, 3 g/m^2, iv weekly for 3 weeks with citrovorum factor rescue; low MTX=methotrexate, 30 mg/m^2, orally, weekly for 3 weeks.



4REGIMEN	ALIVE	DEAD	TOTAL	MEDIAN
—— CAVP/HD M	12	22	34	0.9
- · - CAVP/LD M	10	18	28	0.5
– – CAVPB/HD M	10	9	19	1.7
····· CAVPB/LD M	16	19	35	1.5

FIGURE 3.—Time to treatment failure as a function of initial phase of therapy and methotrexate consolidation. HD M=high-dose methotrexate; LD M=low-dose methotrexate.

Bleomycin also appears to have single-agent activity in non-Hodgkin's lymphoma (*11*). Due to its lack of myelotoxicity, it represents an attractive agent for use in combination drug programs. However, dose-limiting pulmonary toxicity may be more common in non-Hodgkin's lymphoma than in other malignancies and appears at a cumulative dose of 45 to 240 units/m^2 when bleomycin is given at a dose of 15 units/m^2 iv twice monthly (*12*). The antitumor dose response curve for bleomycin has not been established (*13*). For these reasons, it is desirable to use as low a dose of bleomycin as possible while retaining antitumor activity. Since the activity of bleomycin appears greatest against cells in the G2 and M phases of the cell cycle and its serum half-life is short, an increase in efficacy might be anticipated with continuous iv infusion rather than bolus administration (*13*). Furthermore, administration by continuous low-dose infusion may reduce the risk of the pulmonary toxicity. In this regard, a previous CALGB pilot study of 58 patients disclosed no clinical, radiographic, or functional pulmonary toxicity using a dose of 2 units per day for 5 days by continuous iv infusion to achieve steady-state plasma concentrations of bleomycin (*14*).

The initial report by the Southwest Oncology Group of the CHOP (CAVP) combination in histiocytic lymphoma indicated a complete response rate of 68% (*1*). In a subsequent study, a complete response rate of 48% was obtained (*2*). A similar program including bleomycin (BACOP) produced a complete response rate of 48% (*12*). Thus, the complete response rates of 46 to 51% obtained in the current study appear to be consistent with these previous reports. This is particularly true when we consider the impact of the more aggressive restaging employed in this study. The response rate observed here is especially notable since it was obtained in a relatively elderly study population: 50% of the patients were 60 or over with 25% at least 70 years of age (*15*).

This study demonstrates the feasibility of the use of high-dose methotrexate with rescue and the infusion of bleomycin in a cooperative group setting. There was only a slight increase in toxicity attributable to the high-dose methotrexate. However, neither the addition of high-dose methotrexate as used in this study, nor the addition of low-dose bleomycin infusion was shown to lead to significant improvement in complete response rate or failure-free survival in this study. Patients in partial remission after 3 cycles of CAVPB did not have their remission duration increased by the use of high-dose methotrexate. Patients not in complete remission after the first 3 cycles of therapy were not more likely to enter complete remission if high-dose methotrexate was employed. These results do not necessarily imply that the methotrexate does not add to CAVPB in this setting, but raise questions about the advantage of the higher dose. Finally, fewer first relapses involving the central nervous system have thus far been observed among patients who received high-dose methotrexate, a result which merits further follow-up.

REFERENCES

(*1*) Jones SE, Grozea PN, Metz EN, et al: Superiority of adriamycin-containing combination chemotherapy in the treatment of diffuse lymphoma. Cancer 43:417–425, 1979.

(*2*) Armitage JO, Fyfe MAE, Lewis J: Long-term remission, durability and functional status of patients treated for diffuse histiocytic lymphoma with the CHOP regimen. J Clin Oncol 2:898–902, 1984.

(*3*) Coltman CA, Dahlberg S, Jones SE, et al: CHOP is curative in thirty percent of patients with diffuse large cell lymphoma. Proc ASCO 5:197, 1986.

(*4*) Djerassi I, Kim JS: Methotrexate and citrovorum factor rescue in the management of childhood lymphosarcoma and reticulum cell sarcoma (non-Hodgkin's lymphoma). Cancer 38:1043–1501, 1976.

(*5*) Cadman E, Farber L, Berd D, et al: Combination therapy for diffuse histiocytic lymphoma that includes antimetabolites. Cancer Treat Rep 61:1109–1116, 1977.

(*6*) Djerassi I: High-dose methotrexate (NSC M-740) and citrovorum factor rescue: Background and rationale. Cancer Chemother Rep 6:3–6, 1975.

(*7*) Tattersall MH, Parker LM, Pitman SW, et al: Clinical pharmacology of high dose methotrexate (NS-470). Cancer Chemother Rep 6:25–30, 1975.

(*8*) Pitman SW, Frei E: Weekly methotrexate-calcium leucovorin rescue: Effect of alkalinization on nephrotoxicity: Pharmacokinetics in the CNS: and use in the CNS non-Hodgkin's lymphoma. Cancer Treat Rep 61:695–701, 1977.

(*9*) Zuckerman KS, Skarin AT, Pitman SW, et al: High-dose methotrexate with citrovorum factor (MTX/CF) in the treatment of advanced non-Hodgkin's lymphoma (NHL) including CNS involvement. Proceedings of the American Society of Hematology, p. 76, December 1976.

(*10*) Berd D, Cornog J, DeConti RR, et al: Long-term remission in diffuse histiocytic lymphoma treated with combination sequential chemotherapy. Cancer 35:1050–1054, 1975.

(*11*) Crooke ST, Bradner WT: Bleomycin, a review. J Med 7:335–428, 1976.

(*12*) Schein PS, De Vita VT Jr, Hubbard SP, et al: Bleomycin, adriamycin, cyclophosphamide, vincristine and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphomas. Ann Intern Med 85:417–422, 1976.

(*13*) Prestayko AW, Crooke ST: Bleomycin clinical pharmacology. *In* The Bleomycins—Current Status and New Developments (Carter SK, Crooke ST, Umezawa H, eds). New York: Academic Press, 1978, pp 27–40.

(*14*) Ginsberg SJ, Crooke S, Bloomfield C, et al: Cyclophosphamide, doxorubicin, vincristine and low-dose infusion bleomycin in non-Hodgkin's lymphoma: Cancer and Leukemia Group B study 7804. Cancer 49:1346–1352, 1982.

(*15*) Dixon DO, Neilan B, Jones SE, et al. Effect of age on therapeutic outcome in advanced diffuse histiocytic lymphoma: The Southwest Oncology Group Experience. J Clin Oncol 4:295–305, 1986.

Clinical Pharmacodynamic Studies of High-Dose Methotrexate in Acute Lymphocytic Leukemia[1]

William E. Evans,* Minnie Abromowitch, William R. Crom, Mary V. Relling, W. Paul Bowman, Ching-Hon Pui, Judith Ochs, and Richard Dodge[2,3]

ABSTRACT—As the intensity of cancer chemotherapy has been reported to influence clinical response for several drug-sensitive cancers, we have investigated the relation between systemic exposure to high-dose methotrexate (HDMTX) and clinical response in childhood acute lymphocytic leukemia (ALL). A total of 108 consecutive, previously untreated children with "standard-intermediate risk" ALL were randomized to receive postinduction therapy with HDMTX (1000 mg/m^2 iv over 24 hours weekly for 3 weeks, then every 6 weeks for 72 weeks), superimposed on conventional therapy with low-dose 6-mercaptopurine (6MP; 50 mg/m^2 orally per day) and methotrexate (MTX; 25 mg/m^2 orally per week). The systemic clearance of HDMTX ranged from 40 to 131 ml/minute/m^2 among these patients, yielding MTX steady-state plasma concentrations (Cp_{ss}) ranging from 9.3 to 25.4 μM during the infusion. The group of patients ($n=59$) with median MTX $Cp_{ss} < 16$ μM during the HDMTX infusion had a higher probability of having any relapse than patients ($n=49$) with MTX $Cp_{ss} > 16$ μM ($P < 0.05$). In a previously reported univariate analysis, patients with MTX $Cp_{ss} \leq 16$ μM were 3.2 times more likely to relapse on therapy ($P = 0.01$) and 6.9 times more likely to have a hematologic relapse on therapy ($P = 0.001$). Multivariate and stepwise Cox's regression analyses indicated that MTX Cp_{ss} retains its prognostic importance even when other prognostic variables (i.e., DNA Index, WBC, hemoglobin) are considered. When Kaplan-Meier curves of the HDMTX trial (median follow-up > 4 years) are compared to a St. Jude historical control of 258 children with ALL (median follow-up > 7 years) receiving conventional 6MP and MTX maintenance (without HDMTX), only the group of patients with MTX $Cp_{ss} \geq 16$ μM derived statistically significant benefit from the addition of high-dose MTX. This was true for both complete remission and hematologic remission curves. These results demonstrate the potential benefit of adding HDMTX to the maintenance therapy of childhood ALL, but indicate that a dose of 1000 mg/m^2, given as a 24-hour infusion, may not be optimal for patients with reduced systemic exposure due to relatively fast drug clearance.—NCI Monogr 5:81–85, 1987.

The intensity of cancer chemotherapy, measured as either drug dosage or dose rate, has been reported to influence clinical outcome for several drug-sensitive cancers, including breast cancer (*1,2*), Hodgkin's and non-Hodgkin's lymphoma (*3–5*), small cell carcinoma of the lung (*6*), and childhood ALL (*7*). In these previous studies, patients receiving full-dose or a greater percentage of planned chemotherapy responded better than those given half-dose or a lower percentage of planned chemotherapy. For most anticancer drugs, the maximum dose is determined by host tolerance to adverse effects. When leucovorin rescue is given, the dose of MTX is essentially not limited by adverse effects, and therefore can be escalated to obtain optimal therapeutic response. However, the dosages of MTX currently used to treat childhood ALL range from 20 mg/m^2 to 33,600 mg/m^2 per dose (*7–9*) with little evidence as to what dose is most appropriate.

We have previously reported that the rate of MTX clearance can have a significant effect on clinical response in children with "standard-intermediate risk" ALL treated with HDMTX (*10*). We have more recently reported a significantly greater hazard of relapse in those patients whose median MTX plasma concentration was <16 μM during the HDMTX infusion (*11*). The present analysis of this clinical trial updates these results with a longer follow-up and compares these results to those of a large historical control group that received the same maintenance therapy, but without HDMTX.

PATIENTS AND METHODS

HDMTX Group

Every patient with previously untreated "standard-intermediate risk" ALL referred to St. Jude Children's Research Hospital from June 1979 to October 1982 was included in this study, with informed parental consent. The diagnosis of ALL was made from a bone marrow aspirate according to previously described criteria (*12*). For this protocol, standard-intermediate risk ALL patients were defined as those who had at diagnosis a circulating white blood cell count below 100×10^9/liter, lymphoblasts negative for sheep erythrocyte rosette formation and surface immunoglobulin, no lymphoblasts in the CSF, and no thymic mass. Clinical and biological characteristics of acute

---

ABBREVIATIONS: ALL = acute lymphocytic leukemia; Cp_{ss} = steady state plasma concentrations; HDMTX = high-dose methotrexate; 6MP = 6-mercaptopurine; MTX = methotrexate.

---

[1] Supported in part by Leukemia Program Project Grant CA-20180, Cancer Center CORE Grant CA-21765, Grant R01-CA-36401 and by ALSAC.

[2] Pharmaceutical Division, Biostatistics Division, and Department of Hematology Oncology, St. Jude Children's Research Hospital, Memphis, TN.

[3] We are grateful to Drs. Joseph V. Simone, Gaston Rivera, Sharon B. Murphy, and Gary V. Dahl for their suggestions; Ms. C. Riley, S. Treveda, E. Melton, and B. Alexander for their technical assistance; Drs. Clinton Stewart, John Rodman, Paul Hutson, Gary Yee, Michael L. Christensen, Joseph Sinkule, and members of the Pharmaceutical and Nursing Services for their assistance in conducting this study; and Ms. J. Teague for her clerical assistance.

* *Reprint requests to:* William E. Evans, Pharm.D., St. Jude Children's Research Hospital, Clinical Pharmacokinetics/Dynamics Laboratory, 332 North Lauderdale, Memphis, TN 38101.

lymphocytic leukemia patients meeting these criteria have been previously described in detail (*13*). Seventy-three percent of all patients with newly diagnosed ALL referred to this center met these criteria.

All patients received prednisone (40 mg/m^2/day by mouth for 28 days), vincristine (1.5 mg/m^2, maximum 2 mg, iv on days 1, 8, 15, and 22), and L-asparaginase (10^4 IU/m^2 iv on days 4, 8, 12, and 15) induction therapy. When complete remission was documented by means of the bone marrow aspirate, patients were randomized to one of two maintenance treatments: HDMTX with conventional oral 6MP and MTX maintenance therapy (HDMTX Group), or cranial irradiation therapy and sequential chemotherapy without HDMTX. Those patients randomized to the HDMTX group are the subjects of this analysis. Patients treated with HDMTX received iv infusions of MTX (1000 mg/m^2 in 24 hr, weekly for 3 weeks, then every 6 weeks up to 75 weeks) and intrathecal MTX (12 mg/m^2 weekly for 3 weeks, then every 12 weeks for 120 weeks) in addition to conventional ALL maintenance therapy of daily 6MP (50 mg/m^2) and weekly MTX (25 mg/m^2) given by mouth for 120 weeks. Doses of the latter drugs were modified as necessary to maintain white blood cell counts between 2 and 4×10^9/liter. The dosage of HDMTX was the same for all patients throughout therapy. Intravenous hydration (100 ml/m^2/hr, 5% dextrose, and 0.2% sodium chloride) and urinary alkalinization (sodium bicarbonate, 1 g/m^2 by mouth every 6 hr) were initiated in all patients 2 hours before the HDMTX infusion, which was given as a 200-mg/m^2 bolus loading dose followed by an 800-mg/m^2 iv infusion in 5% dextrose, 0.45% sodium chloride at 100 ml/m^2/hour for 24 hours. All patients were hospitalized for their HDMTX infusions, which were administered via an IVAC-230 iv infusion controller. Leucovorin rescue consisted of 30 mg/m^2 calcium leucovorin iv 36 and 42 hours after the infusion started, then 3 mg/m^2 orally at 54, 66, and 78 hours.

Sample collection and analysis.—Blood samples were obtained 24 hours after beginning every MTX infusion. All samples were analyzed for MTX by an enzyme immunoassay which we have previously validated for precision (5% coefficient of variation) and accuracy compared with high-performance liquid chromatography (*14*). A high-performance liquid chromatography assay (*15*) was used to validate the enzyme immunoassay results in a subset of samples and to measure the metabolite 7-hydroxymethotrexate.

Pharmacokinetic analysis.—The systemic clearance of MTX (in ml/min/m^2) was calculated after each infusion from the zero-order iv infusion rate for MTX (0.555 mg/min/m^2) divided by the MTX Cp_{ss} measured at the end of the 24-hour infusion (*16*). Systemic clearance determined in this manner is a measure of all processes (i.e., renal, nonrenal) removing MTX from plasma. The median values of replicate measurements of MTX clearance and of Cp_{ss} were used as the representative values for each patient.

Control Group

The historical control group comprises all children with ALL enrolled on two previous St. Jude protocols (TOTAL VIII and TOTAL IX) (*17,18*) who: a) met the standard-intermediate risk criteria described above for patients in the HDMTX Group, and b) received only 6MP and MTX maintenance. Characteristics of these patients are summarized in table 1. These 258 patients received 4 weeks of prednisone, vincristine, L-asparaginase (± daunorubicin) induction therapy, 2400-rad cranial irradiation and intrathecal MTX (5 doses) as CNS preventive therapy, then 6MP (50 mg/m^2/day orally) and MTX (25 mg/m^2/week iv) as maintenance therapy for 2.5 years. Dosages of 6MP and MTX were adjusted to maintain the WBC count between 2 and 4×10^9/liter.

Evaluation of Response

For both patient groups, remission status was evaluated 4 weeks after initiation of remission induction therapy; complete remission was defined as bone marrow with normal hematopoiesis, and the absence of leukemic lymphoblasts. To document continuous complete remission or diagnose relapse, bone-marrow aspirates and spinal taps were routinely done every 3 months during therapy, then every 4 months for the first year after cessation of therapy, then every 6 months for the second year after cessation of therapy. Hematological relapse and CNS relapse were defined as previously (*12*). For the purposes of this study, hematological relapses included both isolated hematological relapses and those with concurrent CNS or testicular relapse; CNS relapses refer only to isolated CNS relapses.

Statistical Analysis

The probability distributions of remission duration were estimated by the Kaplan-Meier procedure (*19*). The differences in remission status among various groups were com-

TABLE 1.—Summary of patient characteristics[a]

	HDMTX group			Controls
	ALL ($n=108$)	MTX Cp_{ss}, $<16\ \mu M$ ($n=59$)	MTX Cp_{ss}, $\geq 16\ \mu M$ ($n=49$)	($n=258$)
Age				
2–9 years	81	42	39	178
<2, ≥10 years	27	17	10	80
Sex				
girls	47	22	25	130
boys	61	37	24	128
Race				
white	102	55	47	243
black	6	4	2	14
oriental				1
WBC				
$<25 \cdot 10^9$/liter	81	42	39	210
$25\text{–}100 \cdot 10^9$/liter	27	17	10	48
Hb				
≤7 g/dl	54	29	25	116
>7 g/dl	54	30	24	42
DNA index[b]				
≥1.16	30	16	14	NA[c]
<1.16	53	32	21	

[a] Measured at diagnosis.

[b] DNA index (*13*) was determined at diagnosis for only 83 patients in HDMTX group and none of the controls.

[c] NA = not available; DNA index was not routinely measured in trials prior to 1979.

pared by the Breslow test, which is more sensitive to differences evident early in time (*20*). All reported *P*-values are based on 2-tailed statistical tests. In a previous report (*11*), selected clinical and biological variables, in addition to MTX Cp_{ss}, were analyzed individually as single-regression factors in a Cox's proportional hazards model (univariate analysis) (*21*). Multivariate analyses based on Cox's model were used to select a subset of variables most related to remission duration and to assess the effect of adding MTX concentration to the model containing this subset of significant covariates. For the present study, the exact cut-point for dichotomizing MTX steady-state concentrations was based on the minimum *P*-value for the calculated F statistic (*22*).

RESULTS

From July 1979 through November 1982, complete remission was achieved in 220 of 230 children with standard-intermediate risk ALL; of the 216 eligible for randomization, 108 were randomized to the HDMTX Group. Selected clinical and biological characteristics of these patients are summarized in table 1. Additional characteristics of these patients have been previously described in detail (*11*). At the time of this analysis (December 1985), the median length of follow-up for the HDMTX Group was 4.5 years (range: 36 to 78 months). Thus, all patients had completed their HDMTX therapy (15 doses during the first 1.5 years of therapy). No patient developed severe MTX toxicity. MTX systemic clearance averaged 78.4 ml/minute/m^2 (SD = 15.4; range: 40 to 131 ml/minute/m^2) (fig. 1). Interpatient variability in MTX clearance resulted in a wide range of MTX Cp_{ss} during the 24-hour MTX infusion. Median Cp_{ss} for individual patients ranged from 9.3 to 25.4 μM, despite all patients receiving the same dosage of HDMTX. Through December 1985, 37 of 108 patients in the HDMTX Group had relapsed; 24 initial hematologic relapses, 10 initial isolated CNS relapses, 2 initial testicular relapses and 1 renal relapse. Seven patients with initial isolated CNS relapses subsequently developed hematologic relapses. After a median follow-up of >7 years, 139 of 258 patients in the control group have relapsed.

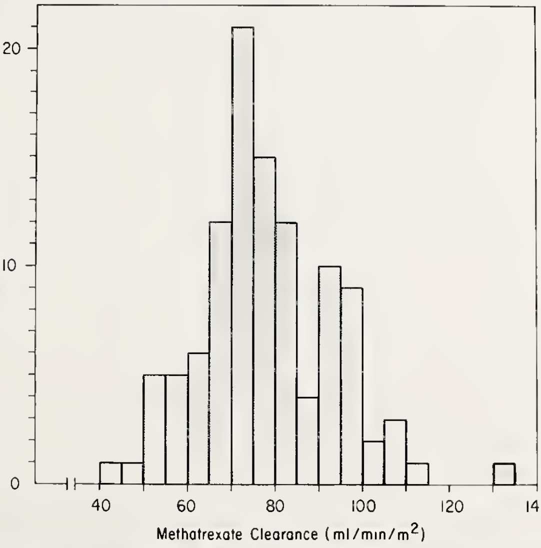


FIGURE 1.—Distribution of patients with HDMTX systemic clearances in each range. Median values of replicate measurements were used for each patient. Reprinted with permission from Clin Pharmacol Ther 39: 592–597, 1986.

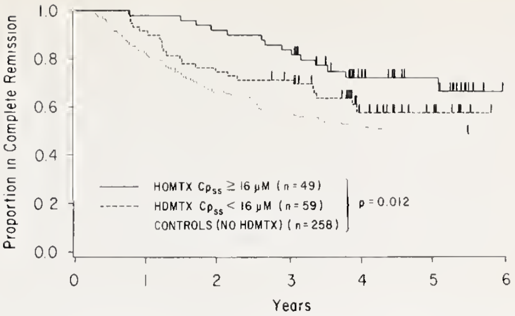


FIGURE 2.—Kaplan-Meier curves of complete remission for patients with median MTX Cp_{ss} <16 μM (- - - -), ≥16 μM (———), and a control group that received no HDMTX (. . . .).

To assess the influence of interpatient variability in HDMTX pharmacokinetics on clinical outcome, the HDMTX Group was divided into two subgroups based on their median MTX Cp_{ss}, as previously described in detail (*11*). In the HDMTX Cp_{ss} <16 μM subgroup, 15 patients had initial hematologic relapses, 6 initial isolated CNS relapses and 2 initial testicular relapses. Five of 6 initial CNS relapses had subsequent hematologic relapses. In the HDMTX Cp_{ss} ≥16 μM subgroup, 9 patients had initial hematologic relapses, 4 initial isolated CNS relapses, 1 renal relapse, and no testicular relapses. Two of 4 initial CNS relapses subsequently developed hematologic relapses. In the control group, 100 had initial hematologic relapses, 21 initial isolated CNS relapses, and 14 initial testicular relapses. Thirteen of 21 patients with initial CNS relapses and 2 of 14 with initial testicular relapses subsequently developed hematologic relapses. When patients with MTX Cp_{ss} <16 μM (n = 59) and patients with MTX Cp_{ss} ≥16 μM (n = 49) were compared to the control group receiving no HDMTX (n = 258), analysis of Kaplan-Meier curves (fig. 2) estimating the probability of complete remission revealed a significant difference among the three groups (Cox-Mantel $P = 0.012$). Pair-wise comparison of each HDMTX subgroup to the control group indicated that only the HDMTX subgroup with MTX Cp_{ss} ≥16 μM was significantly better than the control group (Breslow $P = 0.004$). The estimated proportion of patients in continuous complete remission at 4 years in the HDMTX Cp_{ss} ≤16 μM subgroup, Cp_{ss} ≥16 μM subgroup, and controls was 0.58, 0.74 and 0.50, respectively.

Likewise, analysis of Kaplan-Meier curves (fig. 3) estimating the probability of hematologic remission revealed that only the HDMTX subgroup with MTX Cp_{ss} ≥16 μM was significantly better than controls (Breslow $P = 0.012$).

DISCUSSION

This updated analysis of our previous report (*11*), with an additional year of follow-up, continues to demonstrate a drug concentration–effect relationship for HDMTX in ALL. For the first time, results of the HDMTX protocol are compared to a historical control of 258 patients treated with the same "backbone" of 6MP and MTX, but without

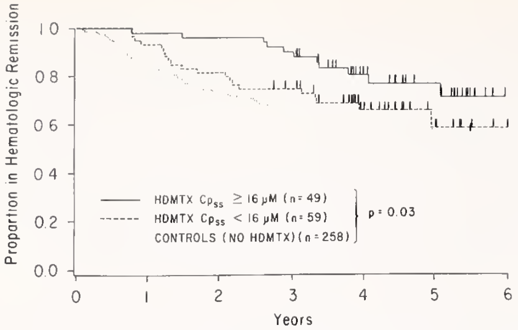


FIGURE 3.—Kaplan-Meier curves of hematologic remission for patients with median steady-state MTX concentrations <16 *μM* (- - - -), ≥16 *μM* (——), and a control group that received no HDMTX (. . . .).

the 15 courses of HDMTX. The present analysis indicates that the entire HDMTX Group (*n*= 108) did significantly better than the control group ($P<0.05$). However, when the HDMTX Group is divided into two subgroups based on the steady-state MTX concentrations achieved during the HDMTX infusions, only the group with MTX $Cp_{ss} \geq 16$ *μM* did significantly better than the controls who received no HDMTX. Although the HDMTX subgroup with MTX $Cp_{ss} < 16$ *μM* was not statistically different from the controls, both complete remission curves and hematologic remission curves fell between the controls and the favorable HDMTX subgroup.

We have recently completed a pharmacokinetic analysis of the 108 patients in the HDMTX group to identify patient-specific characteristics which are related to the interpatient variability in HDMTX systemic clearance. Results of this study (*23*) indicate that interpatient variability in HDMTX clearance, even in children with normal creatinine clearance, can be explained, at least in part, by measures of renal (creatinine clearance) and liver (SGPT) function. These findings are consistent with the known characteristics of MTX elimination (renal and metabolic), but demonstrate that differences in hepatic and renal function influence MTX disposition, and indicate that a portion of the observed variability in HDMTX clearance was nonrandom.

These results demonstrate the potential value of adding HDMTX to the maintenance therapy of standard-intermediate risk ALL in children. Since patients at higher risk of failure (i.e., WBC $>100 \times 10^9$/liter, E-rosette positive lymphoblasts, surface immunoglobulin positive blasts, CNS involvement at diagnosis, or thymic mass at diagnosis) were not included in this study, the importance of MTX concentration in this group cannot be determined. Likewise, it is not known whether the observed concentration–effect relationship for HDMTX would be of comparable importance in a more aggressive multidrug maintenance therapy.

Although the pharmacologic basis for the observed concentration–effect relationship has not been defined, it may relate to several factors, including passive diffusion of MTX across cell membranes in transport-resistant cells (*24–26*), greater accumulation of MTX-polyglutamates at higher extracellular MTX concentrations (*27*) and/or attainment of cytotoxic concentrations in body tissues where MTX is known to distribute relatively poorly (*28*).

Regardless of the pharmacologic basis, these results indicate that the use of HDMTX can have a favorable effect on a subset of children with ALL, but that a dosage of 1000 mg/m^2 given as a 24-hour infusion may not be optimal for those patients with reduced systemic exposure (i.e., Cp_{ss}) due to relatively fast drug clearance.[4] Based on pharmacokinetic data from our pediatric population, a dosage of about 1500 mg/m^2 (i.e., 250 mg/m^2 loading dose plus 1250 mg/m^2 over 24 hours) should yield steady-state MTX concentrations >16 *μM*, even in patients with relatively fast MTX clearance.

REFERENCES

(*1*) BONADONNA G, VALAGUSSA P: Dose-response effect of adjuvant chemotherapy in breast cancer. N Engl J Med 304:10–15, 1981.

(*2*) HIYNIUK W, BUSH H: The importance of dose intensity in chemotherapy of metastatic breast cancer. J Clin Oncol 2:1281–1288, 1984.

(*3*) FREI E III, SPURR CL, BRINDLEY CO, et al: Clinical studies of dichloromethotrexate (NSC29630). Clin Pharmacol Ther 6:160–171, 1965.

(*4*) BRINDLEY CO, SALVIN LG, POTEE KG et al: Further comparative trial of thio-phosphoroamide and mechlorethamine in patients with melanoma and Hodgkin's disease. J Chronic Dis 17:19, 1964.

(*5*) CARDE P, MACKINTOSH FR, ROSENBERG SA: A dose and time response analysis of the treatment of Hodgkin's disease with MOPP chemotherapy. J Clin Oncol 1: 146–153, 1983.

(*6*) COHEN MH, CREAVEN PJ, FOSSIECK JR, et al: Intensive chemotherapy of small cell bronchogenic carcinoma. Cancer Treat Rep 61:349–354, 1977.

(*7*) PINKEL D, HERNANDEZ K, BORELLA L, et al: Drug dosage and remission duration in childhood lymphocytic leukemia. Cancer 27:247–256, 1971.

(*8*) FREEMAN AI, WEINBERG V, BRECHER ML, et al: Comparison of intermediate-dose methotrexate with cranial irradiations for the post-induction treatment of acute lymphocytic leukemia in children. N Engl J Med 308:477–484, 1983.

(*9*) POPLACK DG, REAMAN GH, BLEYER WA, et al: Central nervous system (CNS) preventive therapy with high dose methotrexate (HDMTX) in acute lymphoblastic leukemia (ALL): A preliminary report. Proc Am Soc Clin Oncol 3:204, 1984.

(*10*) EVANS WE, CROM WR, STEWART CF, et al: Methotrexate systemic clearance influences probability of relapse in children with standard-risk acute lymphocytic leukemia. Lancet 1:359–362, 1984.

(*11*) EVANS WE, CROM WR, ABROMOWITCH M, et al: Clinical pharmacodynamics of high-dose methotrexate in acute lymphocytic leukemia: Identification of a concentration–effect relationship. N Engl J Med 314:471–477, 1986.

(*12*) PINKEL D: Five-year follow-up of "total therapy" of childhood lymphocytic leukemia. J A M A 216:648–655, 1971.

---

[4] **Note added in proof:** During the time that has elapsed since this manuscript was prepared for the 1986 symposium, we have had an additional year of follow-up on all patients in this report. Our October 1986 re-analysis of these patients revealed that the differences in both complete remission and hematologic remission Kaplan-Meier curves among the 2 MTX Cp_{ss} subgroups and the controls continue to be statistically significant.

(*13*) Look AT, Roberson PK, Williams DL, et al: Prognostic importance of blast cell DNA content in childhood acute lymphocytic leukemia. Blood 65:1079–1086, 1985.

(*14*) Buice RG, Evans WE, Karas J, et al: Comparison of radioimmunoassay, enzyme immunoassay, and competitive protein binding assay of methotrexate, using HPLC as a standard. Clin Chem 26:1902–1904, 1980.

(*15*) Cairnes DA, Evans WE: High performance liquid chromatographic assay of methotrexate, 7-hydroxymethotrexate, 4-deoxy-4-amino-N^{10}-methylpteroic acid (DAMPA) and sulfamethoxazole in serum, urine and cerebrospinal fluid. J Chromatogr 231:103–110, 1982.

(*16*) Jusko WJ: Guidelines for collection and pharmacokinetic analysis of drug disposition data. *In* Applied Pharmacokinetics: Principles of Therapeutic Drug Monitoring (Evans WE, Schentag JJ, Jusko WJ, eds). San Francisco: Applied Therapeutics Inc, 1980, pp 646–680.

(*17*) Aur RJA, Simone JV, Verzosa MS, et al: Childhood acute lymphocytic leukemia, study VIII. Cancer 42:2123–2134, 1978.

(*18*) Pui C-H, Aur RJA, Bowman WP, et al: Failure of late intensification therapy to improve a poor result in childhood lymphoblastic leukemia. Cancer Res 44:3593–3598, 1984.

(*19*) Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Statist Assoc 53:457–481, 1958.

(*20*) Breslow N: A generalised Kruskal-Wallis test for comparing K samples subject to unequal patterns of censorship. *Biometrika* 57:579–594, 1970.

(*21*) Cox DR: Regression models and life-tables. J Roy Statist Soc B 34:187–202, 1972.

(*22*) Cox DR, Lewis PAW: The statistical Analysis of Series of Events. London: Chapman and Hall Ltd, 1978, p 229.

(*23*) Crom WR, Glynn AM, Abromowitch M, et al: Use of the automatic interaction detector method to identify patient characteristics related to methotrexate clearance. Clin Pharmacol Ther 39:592–597, 1986.

(*24*) Warren RD, Nichols AP, Bender RA: Membrane transport of methotrexate in human lymphoblastoid cells. Cancer Res 38:668–671, 1978.

(*25*) Goldman ID: Membrane transport of methotrexate and other folate compounds: relevance to rescue protocols. Cancer Chemother Rep 6:63–72, 1975.

(*26*) Jolivet J, Cowan KH, Curt GA, et al: The pharmacology and clinical use of methotrexate. N Engl J Med 309: 1094–1101, 1983.

(*27*) Rosenblatt DS, Whitehead VM, Dupont MM, et al: Synthesis of methotrexate polyglutamates in cultured human cells. Mol Pharmacol 14:210–214, 1978.

(*28*) Evans WE, Hutson PR, Stewart CF, et al: Methotrexate cerebrospinal fluid and serum concentrations after intermediate-dose methotrexate infusion. Clin Pharmacol Ther 33:301–307, 1983.

Design and Rationale for Novel Antifolates[1]

J. R. Bertino,[2,3,*] A. Sobrero, E. Mini, B. A. Moroson, and A. Cashmore[3]

ABSTRACT—The goals of new antifolate development are: 1) improved selectivity, 2) improved penetration into pharmacologic sanctuaries, and 3) effectiveness vs. tumors either with intrinsic or acquired resistance to methotrexate (MTX). The major target for antifolate development has been dihydrofolate reductase (DHFR), but other critical folate-dependent enzymes, i.e., thymidylate synthase, methionine synthetase, and folylpolyglutamate synthetase are also important targets for new antifolate development. The possibility that DHFR from tumor tissue differs significantly from normal tissue DHFR now seems improbable, and the ideas of the late Bill Baker to design specific inhibitors of the tumor enzyme vs. the normal tissue DHFR are unlikely to succeed. However, the experience with triazinate (Baker's antifol; TZT) indicates that transport of antifols could be exploited to provide selective toxicity, as well as to provide agents effective vs. MTX-resistant cells. This work led to a second generation of "nonclassical" folate antagonists, of which trimetrexate (JB-11; TMQ) is now in clinical trial. Uptake of TMQ is via an MTX-independent membrane system, and extremely high intracellular levels of this drug are achieved in human leukemia cells.—NCI Monogr 5:87-91, 1987.

The relatively detailed understanding of the pathways and regulation of folate-mediated enzymes, as well as the limited success of MTX in the clinic, has encouraged the search for new antifolates as well as attempts to modulate MTX effects. Figure 1 illustrates some targets that may be useful for new drug development. Although the major focus of antifolate drug development has been the enzyme DHFR, other targets, critical for cell replication, are also worthy of targeting. Folate analogs that inhibit thymidylate synthase are under development with promising early results (*1,2*). Folate analog inhibitors of folate transport, methionine synthetase, and purine biosynthesis are relatively unexplored thus far, and merit additional investigation.

Since inhibitors of these critical targets may also affect normal replicating tissues, the problem becomes one of obtaining more selective tumor cell kill as compared to toxicity to normal stem cells. Some approaches to selectivity that we and others have explored in recent years are listed in table 1. Differential "rescue" using leucovorin, thymidine, or carboxypeptidase G_1 has received a great deal of attention and will not be considered further in this presentation. The reader is referred to published reviews on this topic (*3,4*) and to contributions in this svmposium. Targeting of antifols is part of a more general approach to obtain selective tumor cell kill and will also not be covered in this paper.

EXPLOITING DIFFERENCES IN INTRACELLULAR METABOLISM OF THE FOLATE ANTAGONIST BETWEEN NORMAL AND TUMOR STEM CELLS

The relatively recent discovery that MTX is retained in cells as a consequence of polyglutamylation and the importance of this process for selective antitumor effects of MTX (*5–7*) have led us to consider the development of "prodrugs," i.e., antifolates that are converted to potent inhibitors of enzymes after polyglutamylation (*1,8*). While MTX may be considered to be a prodrug, MTX itself is a potent inhibitor of DHFR and does not require that additional glutamates be added for this activity. However, polyglutamylation does lead to increased retention and effectiveness of this drug as a cytotoxic agent (*5–7*). A more striking example of this concept is 5,8-dideazaisopteroylglutamate (IAHQ), a quinazoline antifolate studied in this laboratory (*1*). This compound is one of a series of 2-amino-4-hydroxyquinazolines synthesized by Dr. John Hynes of the University of South Carolina School of Medicine. We initially screened these compounds as inhibitors of the growth of human colon carcinoma cells (HCT-8) propagated in vitro. IAHQ was the most potent of these compounds, and when tested in mice bearing colon carcinoma No. 38, it was able to cure a substantial number of animals (6/20). In contrast, MTX is relatively ineffective against this tumor. When we evaluated IAHQ as an inhibitor of thymidylate synthase, it proved to be a relatively weak inhibitor of this enzyme (table 2). However, since IAHQ was an excellent substrate for polyglutamate synthetase (*9*), we also tested the ability of the polyglutamate forms of this compound to inhibit thymidylate synthase. A striking increase in potency was observed as additional glutamates were added (table 2). Recent work by Dr. Sobrero and Dr. McGuire has shown that polyglutamates of IAHQ are formed in tumor cells, although the uptake of this compound into these cells is slow and requires a high extracellular concentration for an extended period of time (Sobrero

---

ABBREVIATIONS: DHFR = dihydrofolate reductase; IAHQ = 5,8-dideazaisopteroylglutamate; ID_{50} = the concentration of drug required to inhibit the control enzyme activity by 50%; LD_{10} = a dose that is lethal for 10% of the test subjects; methyl-CCNU = 1-(2-chloroethyl)-3-(3-methylcyclohexyl)-1-nitrosourea; MTX = methotrexate; TMQ = trimetrexate; TZT = triazinate, Baker's antifol.

---

[1] Supported by USPHS Grants CA-08341, CA-08010 and ACS Grant CH253A.

[2] American Cancer Society Professor.

[3] Departments of Medicine and Pharmacology, Yale University School of Medicine, New Haven, CT.

* *Reprint requests to:* J. R. Bertino, M.D., Codirector, Developmental Therapy and Clinical Investigation Program, Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.

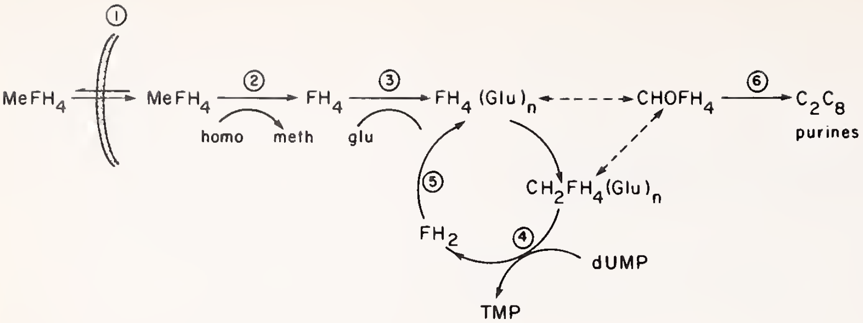


FIGURE 1.—Enzymes in the folate metabolic pathway as possible targets for new antifolates: 1) Folate transport proteins; 2) methionine synthetase; 3) folylpolyglutamate synthetase; 4) thymidylate synthase; 5) DHFR; and 6) purine transformylases.

A, McGuire J, unpublished observations). This drug, especially if it can be modified to enter cells more rapidly, is worthy of further investigation, since it has been shown that marrow precursor cells and gastrointestinal mucosa do not effectively polyglutamate MTX (*10*). Similar studies using IAHQ would be of interest.

EXPLOITING DIFFERENCES IN TRANSPORT BETWEEN NORMAL AND TUMOR STEM CELLS

The most striking example of selectivity of an antifolate drug in a model system is TZT (fig. 2). In rats bearing the Walker 256 carcinosarcoma, doses which are only a fraction of the dose that is lethal for 10% of the test subjects (LD_{10}) are curative; in contrast, maximally tolerated doses of this drug in mice are ineffective (*11*). These disparate responses were found to be due to a marked difference in transport of these two compounds in these cell types. In L1210 cells, propagated either in vitro or in vivo, TZT was taken up poorly, and via the reduced folate-carrier mediated pathway; in W-256 cells uptake was by a different process that resulted in accumulation of high intracellular levels of this drug (*12*). This difference was so striking that inherent sensitivity (or resistance) to this drug could be predicted by measuring the uptake of TZT into the tumor cells after a short-term incubation, or even indirectly by measuring the inhibition of DNA synthesis produced by TZT, using [^{3}H]deoxyuridine as a labeled precursor (table 3). Similar studies were done in blast cells of patients with acute leukemia, and there was little or no difference in uptake noted with TZT when compared to MTX (*13*); and, as predicted, this drug had no therapeutic advantages over MTX in this disease. However, TZT has been shown by the Gastrointestinal Tumor Study Group to have antitumor effects in patients with gastrointestinal malignancies, and the combination of 5-fluorouracil, doxorubicin, and TZT appears to be more active than 1-(2-chloroethyl)-3-(3-methylcyclohexyl)-1-nitrosourea (methyl-CCNU) combined with 5-fluorouracil and doxorubicin in patients with advanced gastric cancer. It would be of interest to use the [^{3}H]deoxyuridine assay to predict response of patients with adenocarcinomas of the gastrointestinal tract to TZT.

Encouraged by the findings with TZT that demonstrated that potent inhibitors of DHFR could be found that were transported differently by various mammalian cells, we screened a series of quinazolines and pyridopyrimidines kindly provided by Dr. E. Elslager of Warner Lambert/Parke Davis (*14*). These compounds were initially synthesized as part of a program to develop antifolate antimalarial agents, and some were highly effective in this screen (*15*). Several of these compounds proved to be highly effective inhibitors of human leukemia DHFR; potent inhibition of the enzyme by certain of these compounds was also reported by McCormack et al. (*16*). On the basis of its potent in vitro effects against several mouse lines, we selected TMQ for further development (fig. 2). This compound was found to be active vs. several mouse tumors, and, based on these studies (*14*) as well as its potent inhibition of human tumor cells in vitro, has entered phase I and II clinical trials.

Since this drug was incapable of being polyglutamylated, we predicted that low-dose continuous exposure over at least 1 generation time would be necessary for inhibition of tumor growth. In some, but not all, tumors tested this hypothesis is proved to be true; however, growth of human colon carcinoma cells (HCT-8) was markedly inhibited by even short-term exposure to high doses of this drug (*17*). Uptake studies showed that as compared to MTX, high intracellular concentrations of TMQ were achieved, and even after washing and resuspending these cells in drug-free medium, sufficiently high intracellular concentrations of the drug were still present to allow continued inhibition of DNA synthesis and cell growth (fig. 3). We are exploring the possible explanations for retention of this drug by HCT-8 cells. Similar studies in tumor cells obtained from

TABLE 1.—Approaches to selectivity with folate antagonists

1. Differential rescue (leucovorin, thymidine, carboxypeptidase G_1, asparaginase).
2. Targeting (monoclonal antibodies, liposomes).
3. Exploiting differences in intracellular metabolism of the folate antagonist between normal and tumor stem cells.
4. Exploiting differences in transport between normal and tumor stem cells.
5. Selective eradication of the drug-resistant cell.

TABLE 2.—Inhibition of thymidylate synthase by IAHQ and its polyglutamate forms

Compound	ID_{50}, μM[a]
IAHQ (H-388)	1.5
IAHQ (glu)[b]	0.17
IAHQ (glu$_2$)	0.05
MTX	30.00

[a] ID_{50} = the concentration of drug required to inhibit control enzyme activity by 50%.

[b] glu = glutamic acid.

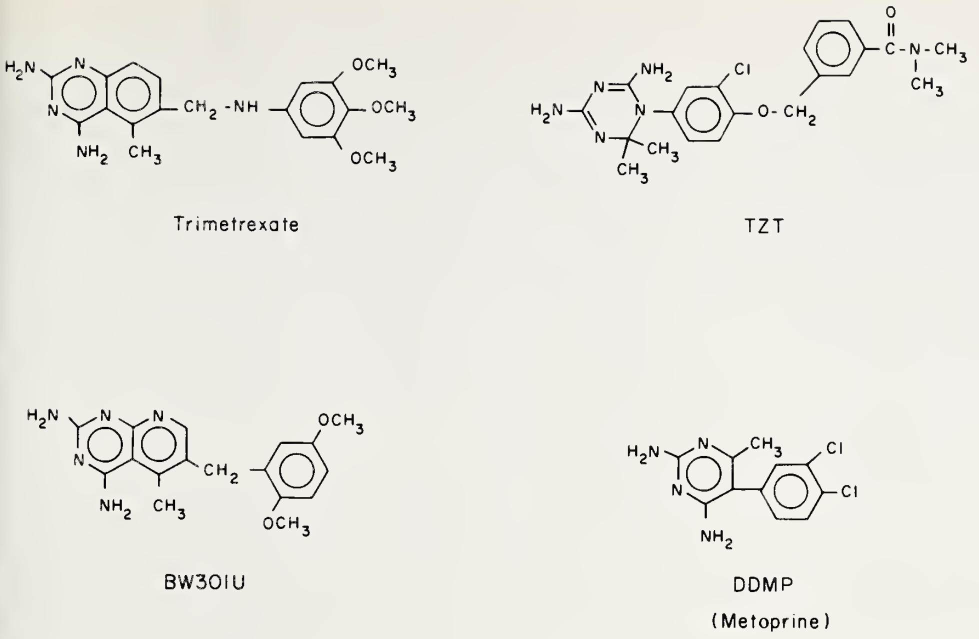


FIGURE 2.—Structures of some "nonclassical" antifolates.

biopsies of human tumors may allow development of a rapid predictive test for tumor cell sensitivity.

DRUG-RESISTANT CELLS AS TARGET FOR DRUG DEVELOPMENT

The current strategy to prevent emergence of drug resistance in the clinic that is being widely tested is to use drug combinations in an alternating fashion. This approach, based on theoretical modeling by Goldie and Coldman (*18*), requires that cross-resistance between the drugs used in combination does not occur, and the combinations used are both equally effective. Except possibly for improved results in Hodgkin's disease and in diffuse lymphoma, the clinical trials to date have not produced evidence of marked benefit.

We have suggested an alternate approach to the problem of drug resistance, namely, the use of a second drug specifically targeted to be cytotoxic to the resistant population, following maximal response to the first drug (*19,20*). Selectivity can be obtained based on an understanding of the genetic basis for, and the phenotypic expression of, these drug-resistant cells (table 4). Thus the second drug employed would depend on the mechanism of resistance encountered after decimating the tumor cell population with the first drug.

Decreased MTX Uptake

For example, since decreased uptake of MTX is a commonly encountered cause for MTX resistance, antifolates that are still powerful inhibitors of DHFR, but enter cells via a different transport mechanism than does MTX, would still be effective vs. MTX-transport-defective resistant cells. Indeed, studies from this and other laboratories have shown that TMQ is still highly cytotoxic vs. MTX-transport-resistant cells (*21–23*). In fact, MTX-transport-resistant CEM cells are collaterally sensitive (more sensitive than the parent line) to TMQ. One possible explanation for this result is that these cells are less able to accumulate folates as well as wild type cells, and as a consequence of decreased intracellular folate pools would be more sensitive

TABLE 3.—Inhibition of [^{3}H]deoxyuridine incorporation into DNA of L1210 leukemia cells or W-256 cells by TZT[a]

Tumor line	Dose, mg/kg	Incorporation of [^{3}H]-deoxyuridine at indicated hr after treatment by TZT, % control	
		1.0	3.0
W-256	6	2.0	4.5
L1210	12	45	65

[a] Reprinted with permission from Cancer Res 33:2972–2976, 1973.

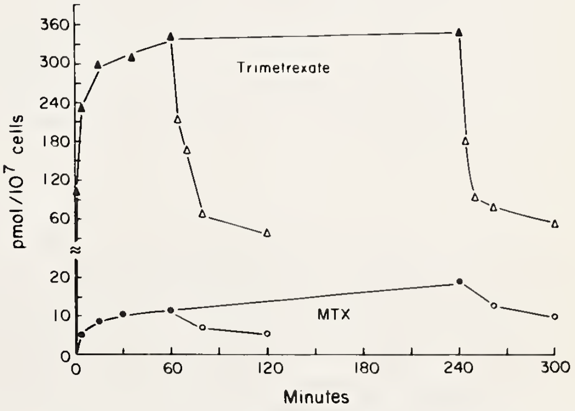


FIGURE 3.—Uptake of [^{14}C]TMQ and [^{3}H]MTX in HCT-8 cells. TMQ or MTX was incubated with HCT-8 cells and uptake measured at the indicated time points (*solid symbols*). At 1 and 4 hr, the cells were washed and resuspended in drug-free medium and efflux measured (*open symbols*).

TABLE 4.—MTX-resistant cells as targets for drug development

Mechanism of resistance	Strategy
I. Decreased uptake	Use of antifolates that do not employ the reduced folate transport system; e.g., TMQ, piritrexim.
II. Gene amplification	Use of antifolates which are substrates for DHFR, and are converted to inhibitors of other key folate enzymes, e.g., 1) homofolate → thymidylate synthase; 2) ? → polyglutamate synthetase; 3) ? → methionine synthetase.
III. Altered DHFR with decreased affinity for MTX	Use of antifolates with greater affinity for altered enzyme.
IV. Decreased polyglutamylation	Antifolates that do not require polyglutamylation for activity, e.g., TMQ, piritrexim.

to inhibitors such as TMQ, or to enzyme- or diet-induced folate depletion. Increased sensitivity of these MTX-transport-resistant cells to enzymatic folate depletion has also been recently shown by our laboratory (*24*). Increased sensitivity of MTX-resistant cells to folate-deficient diets was observed many years ago (*25*) and stimulated our efforts to find an enzymatic method to produce rapid folate depletion.

Gene Amplification

The other common mechanism of drug resistance to MTX observed in experimental as well as human tumor cells is gene amplification (*26,27*). Over 20 years ago, Friedkin suggested and explored the possibility that a folate analog might be synthesized with the property that it could be a substrate for DHFR, and once reduced, an inhibitor of thymidylate synthase (*28*). He was able to show that homofolate and dihydrohomofolate fulfilled these requirements and were effective vs. MTX-resistant mouse tumor sublines with elevated DHFR activity (*29*). Other targets for this approach would be the other target enzymes outlined (vide supra): methionine synthetase and polyglutamate synthetase. While inhibitors with the properties desired are not yet available, this strategy of lethal synthesis, also a prodrug approach, remains an important one for drug development.

Altered DHFR

Although a relatively uncommon explanation for MTX resistance in experimental systems, several cell lines have been described that have altered enzymes with varying degrees of decreased binding to MTX (*19*). With knowledge of the precise point mutation giving rise to the altered enzyme, it may be possible to construct analogs that selectively inhibit the altered enzyme.

STUDIES IN PATIENTS WITH LEUKEMIA

When blast cells are obtained from blood or bone marrow of patients with acute leukemia and their sensitivity to MTX and TMQ tested by the $[^3H]$deoxyuridine uptake assay, in all patients examined to date, TMQ has been more potent than MTX (*30*). Uptake studies have demonstrated that this increased potency correlates with the increased accumulation of TMQ as compared to MTX. In 3 of 5 patients clinically and biochemically resistant to MTX, there was no cross-resistance to TMQ. While the mechanism of resistance to MTX has not been elucidated thus far in these patients, the presumption is that it was due to either impaired uptake of MTX or low level gene amplification. In either circumstance, TMQ may still be expected to be effective therapy for these patients. Thus it may be possible to select patients for TMQ treatment whose tumors are resistant to MTX treatment.

CONCLUSION

Work in folate analog development has progressed rapidly over the past several years, and several second-generation drugs are now in clinical trial. This activity and the promise of new and more selective agents is a good example of what George Hitchings pointed out in 1975 (*31*)—that "historically, most chemotherapeutic agents have been used without an understanding of their mechanism of action. However, the potential rewards of such understanding are enormous, for the practical application of the drug, the discovery of more selective agents, and for fundamental knowledge."

REFERENCES

(*1*) FERNANDES DJ, BERTINO JR, HYNES JB: Biochemical and antitumor effects of 5,8-dideazaisopteroylglutamate, a unique quinazoline inhibitor of thymidylate synthase. Cancer Res 43:1117–1123, 1983.

(*2*) JONES TR, CALVERT AH, JACKMAN AL, et al: A potent antitumor quinazoline inhibitor of thymidylate synthetase. Synthesis, biological properties, and therapeutic results in mice. Eur J Cancer 17:11–19, 1981.

(*3*) BERTINO JR: "Rescue" techniques in cancer chemotherapy: Use of leucovorin and other rescue agents after methotrexate treatment. Semin Oncol 4:203–216, 1977.

(*4*) JOLIVET J, COWAN KH, CURT GA, et al: The pharmacology and clinical use of methotrexate. N Engl J Med 309: 1094–1104, 1983.

(*5*) GALIVAN J, BALINSKA M, NIMEC Z, et al: The glutamylation of methotrexate by tumor cells in vitro following pulse exposure. *In* Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates (Goldman ID, ed). New York: Praeger, 1985, pp 153–159.

(*6*) MCGUIRE JJ, COWARD JK: Pteroylpolyglutamates: Biosynthesis, degradation and function. *In* Folates and Pterins, Chemistry and Biochemistry of Folates (Blakley RL, Benkovic SJ, eds), vol 1. New York: John Wiley and Sons, 1984.

(*7*) MCGUIRE JJ, MINI E, HSIEH P, et al: The role of methotrexate polyglutamates in methotrexate and sequential methotrexate-5-fluorouracil mediated cell kill. Cancer Res 45:6395–6400, 1985.

(*8*) MCGUIRE JJ, MINI E, HSIEH P, et al: Folylpolyglutamate synthetase-relation to methotrexate action and as a target for new drug development. *In* The Development of Target-Oriented Anticancer Drugs (Cheng Y, Goz B, Minkoff M, eds). New York: Raven Press, 1974, pp 97–106.

(*9*) MCGUIRE JJ, HSIEH P, BERTINO JR: Studies on the substrate specificity of mammalian folylpolyglutamate synthetase. *In* Chemistry and Biology of Pteridines (Blair JA, ed). Berlin: De Gruyter, 1983, pp 615–619.

(*10*) Chabner BA, Allegra CJ, Curt GA, et al: The polyglutamylation of methotrexate: Is methotrexate a pro drug? J Clin Inv 76:907–912, 1985.

(*11*) Baker BR: Active site directed irreversible inhibitors of dihydrofolate reductase. Ann NY Acad Sci 186:214–226, 1971.

(*12*) Skeel RT, Sawicki WL, Cashmore AR, et al: The basis for the disparate sensitivity of L1210 leukemia and Walker 256 carcinoma to a new triazine folate antagonist. Cancer Res 33:2972–2976, 1973.

(*13*) Skeel RT, Sawicki WL, Cashmore AR, et al: Inhibition of DNA synthesis in normal and malignant human cells by triazinate (Baker's antifol) and methotrexate. Cancer Res 36:3659–3664, 1976.

(*14*) Bertino JR, Sawicki WL, Moroson BA, et al: 2,4-Diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]-quinazoline (TMQ), a potent nonclassical folate antagonist inhibitor. I. Effect on dihydrofolate reductase and growth of rodent tumors in vitro and in vivo. Biochem Pharmacol 28:1983–1987, 1979.

(*15*) Elslager EF, Johnson JL, Werbel LM: Folate antagonists. 20. Synthesis and antitumor and antimalarial properties of trimetrexate and related 6[(phenylamino)-methyl]-2-4-quinazoline diamines. J Med Chem, in press.

(*16*) Heusner J, McCormack J: Enzymatic assays for 2,4-diamino-5-methyl-6-((3,4,5-trimethoxylanilino)methyl)-quinazoline, a promising new "nonclassical" antifolate. J Pharm Sci 70:827–828, 1981.

(*17*) Mini E, Sobrero A, Moroson BA, et al: Differential efficacy of trimetrexate (2,4-diamino-6-methyl–(3,4,5-trimethoxyanilino methyl) quinazoline, JB-11, TMQ) and methotrexate (MTX) on human colon carcinoma and human leukemia cells in vitro. Proc Am Assoc Cancer Res 26:230, 1985.

(*18*) Goldie JH, Coldman AJ: A model for tumor response to chemotherapy: An integration of the stem cell and somatic mutation hyotheses. Cancer Invest 3:553–564, 1985.

(*19*) Bertino JR, Mini E, Sobrero A, et al: Methotrexate resistant cells as targets for selective chemotherapy. *In* Advances in Enzyme Regulation (Weber G, ed) vol 24. New York: Pergamon Press, 1985, pp 3–11.

(*20*) Sobrero AF, Bertino JR: Alternating trimetrexate (TMQ) with methotrexate (MTX) delays the onset of resistance to antifolates in vitro and in vivo. Proc Am Assoc Cancer Res 27:269, 1986.

(*21*) Mini E, Moroson BA, Franco CT, et al: Cytotoxic effects of folate antagonists against methotrexate-resistant human leukemic lymphoblast CCRF-CEM cell lines. Cancer Res 45:325–330, 1985.

(*22*) Owoshi T, Ohnuma T, Takahashi I, et al: Establishment of methotrexate-resistant human acute lymphoblastic leukemia cells in culture and effects of folate antagonists. Cancer Res 42:1655–1660, 1982.

(*23*) Jackson RC, Fry DW, Boritzki TJ, et al: Biochemical pharmacology of the lipophilic antifolate, trimetrexate. Adv Enzyme Reg 23:187–206, 1984.

(*24*) Sobrero AF, Mini E, Moroson BA, et al: Enhancement of cytotoxic effects of trimetrexate by carboxypeptidase G_2 (CPG_2) on human tumor cell lines. Clin Res 33:578A, 1985.

(*25*) Rosen F, Nichol CA: Inhibition of the growth of an amethopterin-refractory tumor by dietary restriction of folic acid. Cancer Res 22:495–500, 1962.

(*26*) Stark GR, Wahl GM: Gene amplification. Ann Rev Biochem 53:447–491, 1984.

(*27*) Schimke RT: Gene amplification in cultured animal cells. Cell 37:705–713, 1984.

(*28*) Friedkin M: Enzyme studies with new analogs of folic acid and homofolic acid. J Biol Chem 242:1466–1476, 1967.

(*29*) Friedkin M, Cranford ES, Plante LT: Empirical *vs* rational approaches in cancer chemotherapy. Ann NY Acad Sci 186:209–213, 1971.

(*30*) Bertino JR, Mini E, Sawicki WL, et al: Effects of trimetrexate in human leukemia cells from patients sensitive and resistant to methotrexate (MTX). Proc ASCO 4:44, 1985.

(*31*) Hitchings GH: The Beutner Foundation Memorial Award Lecture—Salmon, Butterflies and Cancer Chemotherapy, *In* Pharmacologic Basis of Cancer Chemotherapy. Baltimore: Williams & Wilkins, 1975, pp 25–43.

Methodology for the Rational Development of Methotrexate Analogs in the Clinic

Brian Leyland-Jones,* Peter J. O'Dwyer, Daniel F. Hoth, and Robert E. Wittes[1]

ABSTRACT—This paper suggests a development plan for antifolate analogs, based upon disease classifications that the National Cancer Institute has successfully utilized for the development of anthracycline and platinum analogs. Diseases are classified according to the role of the parent compound in standard therapy: 1) those in which methotrexate is an important component of standard treatment strategies that have state-of-the-art effectiveness; 2) those in which methotrexate has antitumor activity but no clear role in standard state-of-the-art treatment strategies; and 3) those in which methotrexate has insufficient activity to warrant its use alone or in combination. Developmental plans are specified for each of these disease classes. Trimetrexate is used as a specific example for these plans, although this method of drug development is equally applicable to other methotrexate analogs.—NCI Monogr 5:93-97, 1987.

The isolation of novel structures which are active as anticancer agents is a relatively rare event. Based on experience with drugs in other areas of medicine, it is reasonable to suppose that understanding of molecular mechanisms of drug action will allow the development of analogs of greater potency and/or less toxicity. This has occurred with some success in the field of platinum compounds, and progress continues to occur in the area of anthracycline-anthraquinone compounds. With antifolates, the intracellular target is well-defined, though additional determinants may influence selectivity. Such factors include lipophilicity, membrane transport, ability to undergo intracellular metabolism, and hepatic and renal handling. By structural modification of the basic folate molecule, several analogs with widely differing characteristics have been derived.

Many of these analogs have now completed phase I trials and are almost ready for activity testing. The numerous tumors in which antifolates are active form the major target of such trials. However, the population for entry into such studies is not an endless resource, particularly since multiple analogs in various drug classes are coming to the clinic at about the same time. For example, in current clinical development there are some 6 to 8 anthracycline analogs and 6 antifolates. Clearly, to save patient resources and to allow more analogs to be screened more rationally, a highly focused phase III development effort should be considered. This paper proposes a plan for the development of new antifolates based upon our experience with platinum and anthracycline analogs.

GENERAL PRINCIPLES

Analogs are developed for several reasons. The first and most obvious is that the analog may show an activity advantage, either by way of degree of activity, or by way of spectrum of activity. A good example of a compound that is being developed for its in vitro potency advantage is selenazofurin, a selenium-substituted analog of tiazofurin (*1*). 4′-Deoxydoxorubicin is a good example of an anthracycline analog that showed a different preclinical spectrum of activity from its parent compound doxorubicin (*2*). The second criterion for selection of an analog for development is a toxicity advantage. Since very few analogs show marked activity advantage in the preclinical screens over their parent compound, the toxicity advantage has now become the principal reason for selecting compounds for development in the hope that an improved preclinical therapeutic index will lead to better clinical utility. Most of the platinum and anthracycline analogs have been developed for this reason. Third, more favorable formulation characteristics may be a decisive practical advantage. Pentamethylmelamine was developed in the hope (unfortunately not realized) that an iv formulation would bypass the dose-limiting toxicities of hexamethylmelamine (*3*). Other criteria for selection of analogs include preclinical synergism with existing anticancer agents [selenazofurin and 2′-fluoro-5-methyluracil (*4*)] and lack of cross-resistance with the parent compound [trimetrexate (*5*) and tetraplatin (*6*)].

At a time when clinical trial methodology has advanced to the point of demonstrating rigorous statistical differences between related compounds in minimally prior-treated patients, analogs are going into intense competition with one another at both phase II and phase III levels of clinical development. We are now at the stage when we have to be more selective at the preclinical level in the analogs selected for clinical development, and more specific in the questions that we ask of each analog introduced into the clinic. If indeed a compound is brought into the clinic for one specific preclinical reason, for example, tetraplatin based upon its lack of cross-resistance with the parent compound, its phase II and phase III testing should emphasize a test of that hypothesis. Similarly, an analog that shows a tumor-specific increase in spectrum of activity

---

ABBREVIATIONS: CAF = cyclophosphamide, doxorubicin, and 5-fluorouracil; CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; CTF = cyclophosphamide, trimetrexate, and 5-fluorouracil; NCI = National Cancer Institute.

[1] Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

* *Reprint requests to:* Brian Leyland-Jones, M.D., Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Rm. 4C09, Landow Bldg., Bethesda, MD 20892.

over the parent compound should have predominant early screening of that tumor type.

ANTIFOLATE ANALOGS IN THE CLINIC

As the oldest class of antimetabolite, antifolates have had many structural modifications over the past 30 years. Empirical studies of structure–activity relationships yielded analogs which are still being evaluated, such as dichloromethotrexate (*7*) and Baker's antifol (triazinate) (*8*). The future role of such compounds in the clinic is expected to be limited, despite their broad evaluation. A more recent generation of antifolate analogs is represented by the compounds discussed in this symposium. Trimetrexate, piritrexim, and 10-ethyl-10-deaza-aminopterin are in early clinical trials. They have in common that their development was stimulated and assisted by major advances in our knowledge of antifolate-induced cytotoxicity. They attempt to surmount methotrexate resistance that results from impaired transport in different ways. Trimetrexate and piritrexim probably achieve this by entering cells independently of the reduced folate carrier (*9*), while 10-ethyl-10-deaza-aminopterin does so by its greatly enhanced transport by this carrier (*10*). CB-3717 provides a means of addressing methotrexate resistance due to gene amplification of dihydrofolate reductase. This drug has as its major target thymidylate synthase, inhibition of which correlates with cytotoxicity (*11*).

DEVELOPMENT STRATEGY

What is the most rational means of developing antifolate analogs in the clinic? For the 4 compounds discussed above, the ideal method of assessing their mechanisms of bypassing resistance would be to choose tumors resistant to methotrexate on a biochemically defined basis and to test the analog in these tumors. Currently, however, it is not technically feasible to design a clinical trial in this way. Until analysis of tumor cell populations for mechanisms of resistance in real time becomes feasible, these drugs must be evaluated using clinical endpoints. The use of subsets of tumor types in which specific questions may be asked will facilitate their expeditious assessment.

An example of such subset evaluation is provided by the planned clinical development of trimetrexate. Figure 1 demonstrates the strong schedule dependency of trimetrexate in the conventional NCI P388 leukemia screen. The compound is most active when given every 3 hours on days 1, 5, and 9, is very active on the daily 1 through 9 administration, and is essentially inactive on the single-dose administration. These observations provide initial directions as to the clinical development. There is no particular aspect of the screen that suggests areas of potential clinical emphasis (with the possible exception of the good activity of murine colon lines). However, the drug's activity against tumor lines resistant to methotrexate will demand specific clinical trials to test lack of cross-resistance with the parent compound. Moreover, the pronounced schedule-dependency would suggest that initial phase II screening should be conducted on schedules of either frequent or continuous administration; indeed, this may be a perfect compound to test the ability of the preclinical scheduling model to predict optimal clinical schedule for an antimetabolite. We can, therefore, concentrate on two areas: straightforward analog development and scheduling questions. The NCI plans for analog development are discussed here; plans for testing the applicability of the marked preclinical schedule dependency of trimetrexate in the clinic are discussed by O'Dwyer et al (this symposium).

The NCI has already formulated plans for the development of both platinum and anthracycline analogs. As an example, we will discuss the suggested plan for the development of the former. Shown in table 1 are the more common malignancies divided according to cisplatin responsiveness. In the United States, patients with diseases listed in the first category are generally treated with a cisplatin-containing regimen first. Based upon very encouraging preliminary results, especially in ovarian cancer, the question was raised of the activity of the analogs in comparison with front-line cisplatin, with the goal of determining whether the analog has increased activity and/or decreased toxicity compared to the parent compound. Since the major motivation behind carboplatin's development was decreased toxicity, it was decided that the trial design would seek to prove reduced toxicity for analog, while maintaining a pathologic complete response rate within 10% of that of the

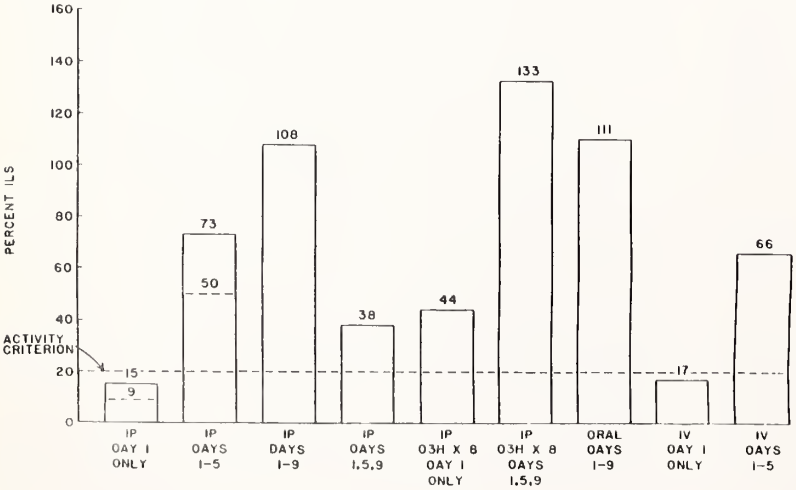


FIGURE 1.—Effect of route and schedule of NSC 352122 (trimetrexate glucuronate) on activity against the ip implanted P388 leukemia. ILS = increased length of survival; Q3H = every 3 hours.

TABLE 1.—Suggestions for phase II clinical trials: Proposals with platinum analogs

Assessment of clinical usefulness in:

1. diseases for which cisplatin is an important component of standard treatment strategies that have state-of-the-art effectiveness:
    - a) Ovarian
    - b) Testicular
    - c) Bladder
    - d) Head and neck
    - e) Small cell lung
2. diseases for which cisplatin has antitumor activity (and perhaps current investigative interest) but no clear role in standard state-of-the-art treatment strategies:
    - a) Colorectal
    - b) Gastric
    - c) Breast
    - d) Non-small cell lung
3. diseases for which cisplatin has insufficient activity to warrant its use alone or in combination:
    - a) Lymphoma
    - b) Renal
    - c) Pancreas
    - d) Melanoma

parent compound in ovarian cancer. Unfortunately, such trials require large numbers of patients (240 per arm) in order to establish the above parameters with 90% power using a 5%-level significance test.

Diseases in the second category of table 1 are those where cisplatin has shown definite activity as defined by shrinkage of tumor, but no definite improvement in survival. In this situation, the best strategy is probably to use an initial phase II trial to demonstrate that the analog has some activity in this disease site and, if that is the case, to move on to a randomized phase III trial against the parent compound as rapidly as possible. This strategy is not very different from that of the first category. These plans may be modified, as follows, to suit the antifolates.

For diseases listed in the third category of table 1, where cisplatin has marginal or no activity at commonly used doses, the question here is to examine the extent of activity of the analogs in the most expedient manner. Since there is no established standard treatment in some of these diseases, many of the patients will have received no prior chemotherapy and have good to excellent performance status. One is therefore able to determine the activity of the analog in a patient population most likely to respond to treatment, and one is also able to greatly expand the database regarding the toxicity associated with the compound.

Tumors in Which Methotrexate Is an Important Component of Standard Treatment Strategies That Have State-of-the-Art Effectiveness

For these tumors the development plan is the most difficult (table 2). The ultimate test of efficacy of trimetrexate in these tumor types is the randomized trial substituting trimetrexate for methotrexate in front-line combinations. Because randomized phase III studies involve large numbers of patients, will usually have survival endpoints, and commit either a cooperative group or several major cancer centers for a multiyear study, the decision to embark upon one is major.

A development plan might be suggested for the example of breast cancer. Phase II trials could be conducted in 3 separate populations of patients with advanced disease: previously untreated patients, cyclophosphamide, methotrexate, and FUra (CMF) failures, and cyclophosphamide, doxorubicin, and FUra (CAF) failures. A number of centers have now found it acceptable to test a new agent for 2 cycles of therapy in previously untreated patients, before initiating standard CMF or CAF therapy. This obviously yields the highest chance of seeing a response, and gives valuable information on the acute toxicity of the agent in previously untreated patients. For the CMF failures, one would have to automatically assume some lack of cross-resistance with the parent compound for a methotrexate analog to be active in this setting.

Based upon the results in phase II, one can then decide whether phase III studies are warranted. If the phase II results have suggested significantly greater activity than might have been expected from methotrexate, then phase III evaluation is an obvious priority. If the drug appears to lack clinical cross-resistance with the parent compound, one might also wish to explore its utility in the salvage situation following failure of the initial combination chemotherapy. If the drug is not obviously more active than methotrexate but appears less toxic, then phase III studies targeted at better tolerance may also be indicated.

In the phase III evaluation, the substitution of analog for parent, such as cyclophosphamide, trimetrexate, and 5-fluorouracil (CTF) versus CMF, is a logical design. As was mentioned in the discussion of platinum analogs, the emphasis in the majority of previous phase III analog trials has been one of reduced toxicity rather than greater activity. If justified by the phase II data, however, we think that the trial design should aim at establishing a higher response rate for the trimetrexate-containing regimen, at equivalent treatment intensities.

Targeting improvement in efficacy, rather than reduced toxicity, is important for 2 reasons. First, from a purely medical point of view, improvement is a more valuable result than therapeutic equivalence. Second, the finding of equivalent efficacy with reduced toxicity in a substitution study is uninterpretable, unless the deleted drug has already been shown to have a role in the combination. Since the contribution of methotrexate to CMF has never been clearly defined, the finding of therapeutic equivalence and reduced toxicity between CMF and CTF could mean either that trimetrexate is as good as methotrexate (and less toxic) or that neither contributes to the efficacy of CMF. If

TABLE 2.—Trimetrexate analog development

Tumors in which MTX[a] is an important component of standard treatment strategies that have state-of-the-art effectiveness:

- a) Lymphoma
- b) Osteosarcoma
- c) Breast
- d) Small cell lung
- e) (Leukemia)

Plan: Phase II single agent
2nd line ---->
Phase II combination ---->
Phase III combination MTX vs. combination TMTX

[a] MTX=methotrexate; TMTX=trimetrexate.

the phase II data do not suggest that targeting increased efficacy is likely to be productive, then toxicity reduction is a reasonable second priority. In either case, however, such trial designs require very large numbers of patients. The current Southwest Oncology Group study in poor-prognosis lymphomas, which examines whether the new intensive, front-line methotrexate-containing regimens are superior to standard cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), require 412 patients per arm in order to detect a 10% difference in response rates (80% versus 70%) with adequate statistical power (0.90) between any 2 treatment arms. To be both ethical and efficient, such a design has to incorporate procedures for early termination of patient accrual to treatment arms with significant inferior response rates.

Tumors in Which Methotrexate Has Antitumor Activity But No Clear Role in Standard State-of-the-Art Treatment Strategies

For these tumors, phase II single-agent testing of a new analog yields little information. The response rate in 30 patients or so may fail to indicate a truly useful advance. It is probably more productive to proceed with either a randomized phase II or phase III trial against the parent compound with an early stopping rule to detect low level of activity of the analog (table 3).

Such trials have already been designed in head and neck cancer for screening highly effective experimental treatment programs against the standard 25% response rate of methotrexate (*12*). An alternative strategy, based upon preclinical observations of synergy, develops a phase II combination of the analog with whichever agent has been shown to be synergistic in vivo, and then takes this combination into a randomized phase III trial versus methotrexate.

We believe that the critical test in this group of tumors is an indication of marked superiority of the single agent in a randomized phase II or III trial.

Tumors for Which Methotrexate Has Insufficient Activity to Warrant Its Use Alone or in Combination

For these tumors (table 4), the clinical plan is relatively straightforward. A conventional phase II single-agent test will screen activity. Generally, 2 phase II trials, which both show no activity in 14 minimally pretreated patients, will discourage any further testing of the compound. If any activity is observed, clinical development can either proceed based upon other drugs which are clinically active in the specific tumor, or based upon preclinical synergy testing in vivo, preferably specific tumor-directed. Further trials will not depend on showing superiority of the analog to methotrexate.

TABLE 3.—Trimetrexate analog development

Tumors in which MTX[a] has antitumor activity but no clear role in standard state-of-the-art treatment strategies:
a) Head and neck
b) Bladder
c) Cervix
Plan: (A) Phase III single agent vs. MTX with early stopping rule
Phase II single agent --> combination phase II -->
Phase III combination vs. MTX

[a] MTX = methotrexate.

TABLE 4.—Trimetrexate analog development

Tumors for which MTX[a] has insufficient activity to warrant its use alone or in combination:

NSCLC[b]	Brain
Colon	Pancreas
Esophagus	Prostate
Stomach	

Plan: Phase II single agent

[a] MTX = methotrexate.
[b] NSCLC = non-small cell lung cancer.

CONCLUSIONS

We would suggest that the rational development of antifolate analogs in the clinic might parallel the disease groupings previously set up for anthracycline and platinum analogs. Two positive aspects of these development plans are: 1) the emphasis on screening analogs for therapeutic activity in the minimally pretreated population; and 2) the emphasis on definitive phase III trials in order to establish the therapeutic index of the drug in responsive tumor types. As an example of the former, the further testing of deoxydoxorubicin and carboplatin in breast cancer was obviated by discouraging results in patients who were not previously treated or were minimally treated previously. Examples of the latter are the various ongoing randomized trials of various anthracycline substitutions for CAF in breast cancer, and the current randomized carboplatin substitution in platinum–cyclophosphamide combinations in front-line ovarian cancer.

Additional advantages of such a model include: 1) it is targeted to specific tumors, building on the probable role of the parent compound in each case; and 2) it focuses the goals of an extremely expensive clinical undertaking. Other fundamental issues which will not be defined by these clinical approaches remain. Unless specifically incorporated into the design of the randomized phase III trials, specific questions concerning lack of clinical cross-resistance with the parent compound, and correlation with preclinical models will not be answered. Mechanistic studies in the clinic are important but difficult. However, without appropriate studies of a drug's cellular pharmacology, the mechanistic advantage defined at the preclinical level cannot be confirmed to correlate with efficacy in the clinic. Hence, the purported rationale for the development of the new agent is neither confirmed nor refuted. The development of anthracycline analogs is witness to this; studies of 4′-epidoxorubicin and deoxydoxorubicin do not indicate where future anthracycline modification should or should not go, and empiricism remains the major influence in this field.

Novel methods for analog evaluation in the clinic are needed. The application of the technology which prompted the development of analog to clinical specimens from patients treated with the analog will maximize the benefits to be obtained from such clinical trials.

REFERENCES

(*1*) BURCHENAL JH, PANCOAST T, CARROL A, et al: Antileukemic, antitumor and cross-resistance studies of 2-β-D-ribofuranosylselanazole-4-carboxamide (selenazofurin). Proc Am Assoc Cancer Res 25:1375, 1984.

(*2*) Casazza AM, Savi G, Pratesi G, et al: Antitumor activity in mice of 4′-deoxydoxorubicin in comparison with doxorubicin. Eur J Cancer Clin Oncol 19:411–418, 1983.

(*3*) Pentamethylmelamine (NSC-118742) Clinical Brochure, Bethesda, MD: Division of Cancer Treatment, National Cancer Institute, 1978.

(*4*) Burchenal JH, Chou TC, Lokys L, et al: Activity of 2-fluoro-5-methylarabinofuranosyluracil against mouse leukemias sensitive to and resistant of 1-β-D-arabinofuranosylcytosine. Cancer Res 42:2598–2600, 1982.

(*5*) Didden SH, Neithammer D, Jackson RC: Patients of cross-resistance to the antifolate drugs trimetrexate, metorprine, homofolate, and CB-3717 in human lymphoma and osteosarcoma cells resistant to methotrexate. Cancer Res 43:5286–5292, 1983.

(*6*) Behrens BC, Hamilton TC, Masuda H, et al: Characterization of a cis-diamminedichloroplatinum (II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Cancer Res 47:414–418, 1987.

(*7*) O'Dwyer PJ, Leyland-Jones BR, Alonso MT, et al: Dichloromethotrexate—a critical reassessment. J Clin Oncol 3:1148–1153, 1986.

(*8*) O'Connell MJ, Schutt AJ, Moertel CG, et al: Phase II clinical trial of triazinate in combination with mitomycin C for patients with advanced gastric cancer. Proc ASCO 5:82, 1986.

(*9*) Jackson RC, Fry DW, Boritzki TJ, et al: Biochemical pharmacology of the lipophilic antifolate trimetrexate. Adv Enzyme Regul 22:187–206, 1984.

(*10*) Sirotnak FM, DeGraw JI: Selective antitumor activity of folate analogs. *In:* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WB, et al, eds). Orlando: Academic Press, 1984, pp 43–95.

(*11*) Jackson RC, Jackman AL, Calvert AH: Biochemical effects of a quinazoline inhibitor of thymidylate synthetase *N*-(4-(*N*((2-amino-4-hydroxy-6-quinazolinyl)methyl)prop-2-ynyl-amino)benzoyl)-L-glutamic acid (CB3717) on human lymphoblastoid cells. Biochem Pharmacol 32:3783–3790, 1983.

(*12*) Eisenberger MA, Ellenberg SS, Leyland-Jones BR, et al: Advantages of early stopping rules for randomized trials (RT) in head and neck cancer. Proc ASCO 4:134, 1985.

Therapeutic Synergy of Trimetrexate (CI-898) in Combination With Doxorubicin, Vincristine, Cytoxan, 6-Thioguanine, Cisplatin, or 5-Fluorouracil Against Intraperitoneally Implanted P388 Leukemia

Wilbur R. Leopold,[1,*] Donald J. Dykes,[2] and Daniel P. Griswold, Jr.[2]

ABSTRACT—**In order to support planning of clinical trials of trimetrexate (CI-898) in combination chemotherapy regimens, several studies were carried out to determine the potential for therapeutic synergy between CI-898 and several clinically proven agents including doxorubicin, cytoxan, FUra, 6-thioguanine (6-TG), vincristine, methotrexate, and cisplatin. F_1 hybrid mice bearing either early or advanced stage P388 leukemia were treated with either the single agents or with various combination regimens and observed for lifespan. These studies demonstrated a high degree of therapeutic synergism between CI-898 and doxorubicin, cytoxan, and 6-TG. Combinations of CI-898 with FUra, vincristine, and cisplatin also produced enhanced tumor cell kill, although the results were less dramatic than those mentioned above. The host toxicities of CI-898 and methotrexate were synergistic and appeared to negate any potential therapeutic benefit of the combination. The synergistic effect of the doxorubicin, vincristine, and cytoxan combinations may have been due to a lack of overlapping host toxicities, but the synergy observed with 6-TG was obtained in spite of synergistic host toxicity.—NCI Monogr 5:99–104, 1987.**

Trimetrexate, which has also been known as CI-898, JB-11, and TMQ, is a nonclassical folate antagonist first synthesized by Elslager et al. (*1*). In preclinical tumor models, trimetrexate demonstrated a spectrum of activity superior to that of methotrexate. Trimetrexate had confirmed activity against P388 and L1210 leukemias, B16 melanoma, the CD8F$_1$ mammary carcinoma, colon adenocarcinomas 26, 36, and 38, and the M5076 sarcoma (*1–7;* Leopold WR, unpublished results). Trimetrexate was inactive against Lewis lung carcinoma and against the MX-1, CX-1, and LX-1 human tumor xenografts in nude mice.

Biochemically, trimetrexate is a tightly binding inhibitor of dihydrofolate reductase (*3,6*) that enters cells by a pathway different from that of methotrexate (*8*). As such it retains activity in vitro against cell lines resistant to methotrexate by virtue of a transport defect but not against those that overproduce the target enzyme (*3,8–10*). Studies of the cell cycle effects of trimetrexate demonstrated that it is highly cytotoxic to S-phase cells but not to cells in other portions of the cell cycle (*4*). The turnover of trimetrexate in cells is rapid and the half-life in dogs is approximately 3.5 hours (*11*). Thus, unlike methotrexate or metaprine, optimal treatment schedules for trimetrexate employ highly divided dose regimens that can better maintain cytotoxic plasma levels for sustained periods of time (*2,4*).

Its superior spectrum of anticancer activity, activity against some methotrexate-resistant cell lines, and its good bioavailability (*2,11*) prompted the development of trimetrexate to clinical trial. Combination, rather than single-agent, regimens are the clearly indicated choice for most clinical cancer chemotherapy. The rationale for combination regimens is derived from such factors as variable fractions of specifically drug-resistant tumor cells, tumor heterogeneity, and severe dose-limiting toxicities (i.e., poor selectivity) of the anticancer agents in current use. Thus, the potential for success of any new agent in the cancer clinic depends not only upon its degree and spectrum of activity as a single agent, but also upon its ability to interact favorably with other anticancer drugs in combination chemotherapy protocols. Initial findings of our studies of the anticancer activity of trimetrexate in combination with clinically proven agents are presented here.

MATERIALS AND METHODS

The general methods employed for these studies have been previously described (*12,13*). All mice were quarantined for at least 1 week prior to allocation to test groups. P388 leukemia was passaged weekly in DBA2 mice according to the standard National Cancer Institute protocol (*14,15*). On day 0 of a given test, all mice for the test were randomized, injected ip with suitably diluted ascites fluid from P388 leukemia-bearing donor mice, and rerandomized to test cages for subsequent treatment. The initial inoculum for all but the tumor titration groups was one million cells as determined by hemocytometer count. Each experiment contained an internal control and tumor titration that enabled the determination of the tumor doubling time, estimated tumor cell kill ($\log_{10}$), and the number of tumor cells surviving treatment (*13*).

Drug treatment was begun on either day 1 or day 4 depending on the relative activity of the agents to be tested. Trimetrexate was administered ip every 3 hours for 24 hours on days 1, 5, and 9 or days 4, 8, and 12 as indicated. Doxorubicin was given iv on days 1, 5, and 9; 6-TG was given ip daily for 9 days. Methotrexate was administered ip on days 4, 8, and 12. FUra was given ip daily for 9 days. Cytoxan was given ip on days 4, 8, and 12. Vincristine was administered iv on days 1, 5, and 9. Cisplatin was given ip on days 4, 8, and 12. All of the tested drugs were prepared for injection as indicated. All drugs were injected within

---

ABBREVIATIONS: CTI = combination toxicity index; %ILS = percent increase in host lifespan; LD$_{10}$ = dose which is lethal to 10% of animals; 6-TG = 6-thioguanine.

[1] Warner-Lambert/Parke-Davis Pharmaceutical Research, Ann Arbor, MI.

[2] Southern Research Institute, Birmingham, AL.

[*] *Reprint requests to:* Wilbur R. Leopold, Ph.D., Warner-Lambert/Parke-Davis Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor, MI 48105.

1 hour of dissolution with the exception of trimetrexate, which was made up twice each day. Diluents used for each of the drugs were as follows: trimetrexate—distilled water; FUra, cytoxan, vincristine, doxorubicin, and cisplatin—saline; 6-TG—saline + base; methotrexate—2% sodium bicarbonate. All mice were treated on the basis of cage average weight. In combination regimens, trimetrexate was given approximately 30 minutes before the clinically used agent when the clinically proven agent was to be given ip. When the clinically used drug was to be given iv, trimetrexate was given immediately (within 1 min) after the other drug. On days in which trimetrexate was given, the clinically used agents were given as described above with the first trimetrexate treatment of the day.

Group average weights were determined for all groups on day 1 and on all treatment days in each experiment. Weight change was calculated as the difference in mean group weights on the first and last days of treatment. Animals were monitored for lifespan. Calculations of median day of death, percent increase in host lifespan (%ILS), number of cells alive at the end of the last treatment, and logarithm of net tumor cell kill (change in tumor burden at last treatment) employed standard methods (*12,13*). Tumor-free survivors were excluded from these calculations. Thus, these end points reflect the effect of the therapy on treatment failures.

Therapeutic synergism is defined for these studies as follows: Two drugs are therapeutically synergistic if a combination regimen produces a significantly better tumor cell kill than either of the single agents alone at optimal or maximum tolerated doses. If the single-agent therapy fails to produce a clear-cut optimal response, conclusions regarding therapeutic synergy are tenuous at best. For these studies, differences in tumor cell kill of less than 1 decade are not likely to be significant.

Since necropsy data were not obtained, deaths were assumed to be due to drug toxicity if, in an otherwise active treatment regimen, an animal died at a significantly earlier date than its cagemates that received similar treatment.

The data obtained from these experiments enable a crude assessment of the relative overlap of host toxicities of the test agents by determination of the Combination Toxicity Index (CTI) (*13,16*). This type of analysis is best done in nontumor-bearing mice. However, the analysis can be carried out in tumor-bearing mice with maximum tolerated doses estimated from the death patterns observed or with the maximum tolerated dose assumed to be the observed optimal dose. Both of these methods were used for these studies where possible. For determination of the CTI, the maximum tolerated dose, or the dose which is lethal to 10% of the animals (LD_{10}), was first estimated for each agent. The CTI for the combination is the sum of the fractional LD_{10} of the single agents used in the maximum tolerated combination regimen. For example, if the LD_{10} of compounds A and B are 10 and 20, respectively, and the doses of A and B employed in the optimal combination regimen are 6 and 10, respectively, the CTI would be calculated as:

$$\underset{\text{Drug A}}{(6/10)} + \underset{\text{Drug B}}{(10/20)} = 1.1 = \text{CTI}$$

CTI values near 1.0 indicate that the host toxicities of the tested agents are roughly additive and that significant overlap of target organs for dose-limiting host toxicity is likely. CTI values between 1.5 and 2.0 suggest that the two agents have non-overlapping host toxicities and that near maximum tolerated doses of each agent can be given in the combination without increased toxicity. Finally, CTI values significantly less than 1.0 suggest that the drugs in the combination are synergistic with respect to host toxicity, while values greater than 2.0 indicate that the drugs are antagonistic with respect to host toxicity.

RESULTS

Studies of the combinations of trimetrexate with doxorubicin, FUra, vincristine, and 6-TG were carried out against early-stage P388 leukemia (4 to 5×10^6 cells at first treatment) (table 1), while studies of trimetrexate in combination with methotrexate, cytoxan, or cisplatin were against more advanced leukemia (about 1×10^8 cells at first treatment) (table 2). This was done to avoid curative therapy by methotrexate, cytoxan, or cisplatin as single agents, which would preclude the demonstration of therapeutic synergy.

Trimetrexate + Doxorubicin

In the first trial, the combination of CI-898 with doxorubicin on four different dose regimens (31 and 4, 31 and 2.7, 20 and 4, 20 and 2.7 mg/kg per injection, respectively) produced approximately 0.7 logs better net tumor cell kill than the best of the single agents, respectively (CI-898 at 31 mg/kg per injection). Doxorubicin as a single agent was somewhat less active than expected based on historical data, but the test was technically satisfactory. In the confirmatory trial, all 6 of the combination regimens tested produced better tumor cell kill than either of the single agents. The optimal combination regimen (trimetrexate at 46 mg/kg per injection and doxorubicin at 6 mg/kg per injection) produced 5.6 logs greater tumor cell kill than the best of the single agents. This test was flawed by failure to reach a clear optimal dose for doxorubicin as a single agent. However, the weight loss (2 g) observed at the highest dose of doxorubicin tested, combined with a large amount of historical data, suggests that lifespan would not be significantly increased at higher dosage levels. These studies indicate that the combination of trimetrexate with doxorubicin is therapeutically synergistic, producing an average of 3 logs better tumor cell kill than optimal doses of either of the single agents.

Trimetrexate + FUra

In the initial study of CI-898 in combination with FUra, several of the combination regimens were superior to either of the single agents, with 2 of the combination regimens reducing the estimated tumor burden to a single cell. Thus, the optimal combination regimens produced 2.1 logs greater tumor cell kill than the most active of the single agents (FUra). A second test confirmed these findings with several of the combination regimens yielding net tumor cell kill 1.3 logs greater than FUra alone. Both trials were technically satisfactory. The average degree of synergy for the two studies was 1.7 logs.

Trimetrexate + Vincristine

This combination also demonstrated synergy in both of the tests done to date. In our first trial, all 5 of the tolerated

TABLE 1.—Response of intraperitoneally implanted P388 leukemia to trimetrexate, doxorubicin, FUra, vincristine, and 6-TG as single agents and in combination

	Test 1[a]				Test 2			
Treatment[b]	Dose per injection mg/kg	Median survival days	% ILS (Cured[c]/treated)	$\log_{10}$ net change in tumor burden	Dose per injection mg/kg	Median survival days	% ILS (Cured[c]/treated)	$\log_{10}$ net change in tumor burden
Control		10.5	(0/30)			11.0	(0/30)	
CI-898	70	4.0	−62	Toxic	70	17.5	59	+1.4
	46	21.0	100 (1/10)	−1.0	46	21.0	90	−0.6
	31	23.0	119 (2/10)	−2.4	31	21.0	90	−0.6
	20	22.5	114	−2.1	20	21.5	95	−1.0
					13	19.5	77	+0.3
DOX	9.0	11.5	9	+2.0	9.0	20.5	86	−1.0
	6.0	11.0	4	+2.0	6.0	15.0	36	+1.6
	4.0	12.0	14	+1.9	4.0	14.0	27	+1.8
	2.7	11.0	4	+2.0	2.7	12.0	9	+2.1
FUra	45	11.0	4	+2.0	45	11.0	0	+2.2
	30	21.5	104	−2.1	30	24.5	122	−3.5
	20	25.0	138	−4.5	20	21.5	95	−1.6
	13	22.0	109	−2.4	13	18.0	63	+0.6
VCR	2.0	18.5	76	0	2.0	21.5	95	−1.6
	1.3	19.0	80	−0.3	1.3	18.0	63	+0.6
	0.89	16.0	52	+1.4	0.89	14.0	27	+1.8
	0.59	14.0	33	+1.7	0.59	14.5	31	+1.7
6-TG	3.0	15.0	42	+1.5	3.0	17.0	54	+1.3
	2.0	17.0	61	+1.0	2.0	17.0	54	+1.3
	1.3	16.0	52	+1.4	1.3	15.0	36	+1.6
	0.89	15.0	42	+1.5	0.89	13.0	18	+1.9
CI-898+DOX	46+6.0	15.5	47	+1.6	46+6.0	31.0	181 (1/10)	−6.6
	31+4.0	24.0	128	−3.1	31+4.0	26.0	136	−3.8
	31+2.7	24.0	128	−3.1	31+2.7	23.5	113	−2.2
	20+4.0	24.0	128	−3.1	20+4.0	27.0	145 (1/10)	−4.5
	20+2.7	24.0	128	−3.1	20+2.7	25.0	127	−3.2
	13+1.8	22.0	109	−1.7	13+1.8	22.0	100	−1.3
CI-898+FUra	46+30	8.0	−24	Toxic	31+20	18.5	68	+1.0
	31+20	10.0	−5	+2.3	31+13	27.0	145 (1/10)	−4.5
	31+13	26.0	147	−4.5	20+20	22.0	100	−1.3
	20+20	10.5	0	+2.2	20+13	27.5	150	−4.8
	20+13	29.0	176 (1/10)	−6.6	13+13	27.5	150	−4.8
	13+9	29.0	176	−6.6	13+9.0	23.0	109	−1.9
					8.7+6.0	22.0	100	−1.3
CI-898+VCR	46+1.3	7.0	−34	Toxic	46+1.3	20.0	81	0
	31+0.89	25.5	142	−4.2	31+0.89	25.0	127	−3.2
	31+0.59	24.0	128	−3.1	31+0.59	24.0	118	−2.5
	20+0.89	26.0	147	−4.5	20+0.89	24.5	122	−2.9
	20+0.59	25.0	138	−3.8	20+0.59	24.0	118	−2.5
	13+0.39	25.0	138	−3.8	13+0.39	22.0	100	−1.3
CI-898+6-TG	46+2.0	7.5	−29	Toxic	31+0.89	25.5	131	−3.5
	31+1.3	10.5	0	+2.2	20+1.3	16.0	45	+1.9
	31+0.89	12.0	14	+2.0	20+0.89	14.5	31	+1.9
	20+1.3	11.0	4	+2.2	13+0.89	22.5	104	−1.6
	20+0.89	11.0	4	+2.2	13+0.59	27.5	150	−4.8
	13+0.59	26.5	152	−4.9	8.7+0.59	26.0	136	−3.8
					8.7+0.39	23.5	113	−2.2

[a] Estimated tumor burdens at first treatment were 4.9×10^6 cells for test 1 and 4.3×10^6 cells for test 2. Estimated tumor doubling times were 0.43 and 0.47 days for tests 1 and 2, respectively.

[b] Treatment regimens were as described in text. CI-898 = trimetrexate; DOX = doxorubicin; VCR = vincristine.

[c] Cures are 60-day survival for test 1 and 40-day survival for test 2. Cures are excluded from the calculations of median lifespan, %ILS, and net reduction in tumor burden.

combination regimens studied produced better reductions in tumor burden than trimetrexate, which was the most active of the single agents. The most active of the combination regimens reduced the estimated tumor burden surviving the last treatment to just 150 cells, a 2.1 log better cell kill than that obtained by optimal treatment with trimetrexate. Similar results were obtained in the second study, in which vincristine was the most active of the single agents. Although a clear-cut optimal dose was not obtained for vincristine in the second trial, significant improvement at

TABLE 2.—Response of intraperitoneally implanted P388 leukemia to trimetrexate, cisplatin, methotrexate, and cytoxan as single agents and in combination

	Test 1[a]				Test 2[a]			
Treatment[b]	Dose per injection mg/kg	Median survival days	% ILS (Cured[c]/treated)	Log$_{10}$ net change in tumor burden	Dose per injection mg/kg	Median survival days	% ILS (Cured[c]/treated)	Log$_{10}$ net change in tumor burden
Control		11.0	(0/30)			11.0	(0/30)	
CI-898	70	6.5	−31	Toxic	70	9.0	−19	Toxic
	46	18.5	68	+0.2	46	20.5	86	0
	31	22.5	104	−1.2	31	18.5	68	+0.2
	20	20.5	86	−0.2	20	20.0	81	0
					13	19.0	72	+0.1
DDP	10	17.0	54	+0.3	10.0	24.0	118	−2.8
	6.7	27.5	150	−4.4	6.7	19.0	72	+0.1
	4.4	19.5	77	−0.2	4.4	16.5	50	+0.3
	3.0	16.0	45	+0.5	3.0	12.0	9	+0.9
CTX	130	22.0	100 (1/10)	−1.5	130	23.0	109 (1/10)	−2.1
	87	22.0	100	−1.5	87	25.5	131	−3.5
	58	23.0	109	−2.0	58	20.5	86	0
	38	23.0	109	−2.0	38	20.5	86	0
MTX	4.0	15.5	40	+0.5	4.0	20.0	81	−0.1
	2.6	19.0	72	0	2.6	19.5	77	0
	1.8	18.5	68	0	1.8	17.0	54	+0.3
	1.2	18.0	63	+0.2	1.2	15.0	36	+0.5
					0.8	14.5	31	+0.6
CI-898+DDP	46+6.7	10.5	−5	Toxic	31+3.0	26.5	140	−3.8
	31+4.4	17.0	54	+0.3	20+4.4	28.0	154 (1/10)	−4.8
	31+3.0	16.0	45	+0.6	20+3.0	29.0	163	−5.5
	20+4.4	24.0	118	−2.0	13+3.0	26.0	136	−3.6
	20+3.0	29.5	168	−4.9	13+2.0	22.5	104	−1.1
	13+2.0	26.0	136	−3.1	8.7+1.3	23.0	109	−1.5
CI-898+CTX	46+87	14.0	27 (1/10)	+0.9	46+87	25.0	127 (9/10)	−2.8
	31+58	25.0	127	−2.6	31+58	25.0	127	−2.8
	31+38	33.5	204	−7.0	31+38	31.0	181 (1/10)	−6.9
	20+58	36.5	231 (2/10)	−8.1	20+58	22.0	100	−0.8
	20+38	33.5	204	−7.0	20+38	31.0	181	−6.9
	13+25	29.5	168	−4.9	13+25	27.0	145	−4.2
CI-898+MTX	46+2.6	9.0	−19	Toxic	20+1.2	22.0	100	−0.8
	31+1.8	9.0	−19	Toxic	13+1.2	13.5	22	+0.8
	31+1.2	9.0	−19	Toxic	13+0.8	21.5	95	−0.4
	20+1.8	9.5	−14	Toxic	8.7+0.8	23.0	109	−1.5
	20+1.2	9.0	−19	Toxic	8.7+0.5	21.0	90	−0.1
	13+0.8	13.0	18 (1/10)	+1.0	5.6+0.5	20.5	86	0
					5.6+0.3	18.5	68	+0.2

[a] Estimated tumor burdens at first treatment were 1.1×10^8 cells for test 1 and 1.4×10^8 cells for test 2. Estimated tumor doubling times were 0.57 and 0.45 days for tests 1 and 2, respectively.

[b] Treatment regimens were as described in text. CI-898=trimetrexate; DDP=cisplatin; CTX=cytoxan; MTX=methotrexate.

[c] Cures are 60-day survival for test 1 and 40-day survival for test 2. Cures are excluded from the calculations of median lifespan, %ILS, and net reduction in tumor burden.

higher dosages is not likely based on the weight loss at the highest dose tested (5 g). The average degree of synergy between trimetrexate and vincristine for these trials was 1.8 logs.

Trimetrexate + 6-TG

Marked synergy was observed between trimetrexate and 6-TG in both of the trials to date. In the initial study, the optimal combination regimen reduced the tumor burden to approximately 70 cells. The tumor cell kill was 2.5 logs better than that with trimetrexate alone at its optimal dose. In addition the best combination regimen in that test was that with the lowest doses tested. It was at least possible that still greater synergy might be obtained at lower dosages. In the second test, synergy for this combination was confirmed. For this second trial, dose levels in the combination regimens were lowered while the doses used for evaluation of the single agents were the same as those for the initial study. The optimal combination regimen once again reduced the tumor burden to about 70 cells, a 3.8 log better tumor cell kill than that obtained with the single agents. This trial was flawed by the failure to demonstrate a clear-cut optimal dose of 6-TG, but a plateauing of the dose response, which often indicates that the optimal response would be obtained at intermediate dose levels, was noted. Thus, marked synergy between 6-TG and trimetrexate was confirmed. The average degree of synergy observed was 3.2 logs.

Trimetrexate + Cisplatin

The results of the initial test indicated that the combination of these agents did not produce significantly better cell kill at tolerated doses than the single agents alone. The best of the combination treatments produced only 0.5 logs better cell kill than the optimal dose of cisplatin. In the second trial, cisplatin was considerably less active as a single agent and an optimal dose level was not reached for cisplatin. Thus, although the optimal combination regimen produced 2.7 logs better cell kill than either of the single agents, the existence of therapeutic synergy between these agents is likely but not certain. The average degree of synergy observed was 1.6 logs.

Trimetrexate + Cytoxan

The combination of trimetrexate with cytoxan was highly synergistic in both of the studies to date. In the first study, 3 of the 6 combination regimens studied reduced the tumor burden to 10 cells or less. The optimal combination produced more than 6 logs better cell kill than cytoxan alone. In the second test, the optimal combination regimen cured 9 out of the 10 treated mice, and 2 of the other combination regimens tested reduced the tumor burden to less than 20 cells.

Trimetrexate + Methotrexate

In the initial study of this combination, trimetrexate and methotrexate appeared to be synergistic with respect to host toxicity. Because of the marked toxicity of this combination, an assessment of therapeutic synergy was not possible. However, the death pattern of the mice treated with the lowest combination dose tested suggested that some therapeutic gain might be demonstrated for the combination at still lower doses, and a second test was carried out with altered combination regimens. Results of the second trial indicated that some, although slight, benefit might be obtained from the combination in spite of the enhanced host toxicity. The optimal combination produced 1.4 logs better tumor cell kill than the single agents. However, this test was flawed by failure to reach an optimal dose of methotrexate alone. Because of this flaw and because of the only marginally significant difference between the best single agent and the combination regimen, it is not possible to conclude that CI-898 and methotrexate are therapeutically synergistic with the treatment schedules and sequences employed for these tests.

Table 3 summarizes the overall results of all of the efficacy trials described in this report.

CTI

The estimated CTI values for the combinations studied in these experiments are summarized in table 4. Host toxicities for trimetrexate and vincristine, doxorubicin, or cytoxan appeared to be partially nonoverlapping, as the calculated CTI values for these combinations were all about 1.5 or greater. Combinations of trimetrexate with FUra and cisplatin appeared additive with respect to host toxicity. The CTI values for the combinations of CI-898 with methotrexate or 6-TG were significantly less than 1.0, indicating synergistic host toxicity for these combinations. It is interesting that the combination of trimetrexate with 6-TG demonstrated therapeutic synergy at tolerated doses in spite of the enhanced host toxicity of the combination.

TABLE 3.—Summary of CI-898 combination chemotherapy studies. Degree of observed therapeutic synergism

Combination[b]	Degree of synergy[a] Test 1	Test 2	Mean
Early stage P388 leukemia			
CI-898 + DOX	0.7	3.5–5.6	2.1–3.2
CI-898 + FUra	2.1	1.3	1.7
CI-898 + VCR	2.1	1.6	1.8
CI-898 + 6-TG	2.5	3.8	3.2
Advanced stage P388 leukemia			
CI-898 + DDP	0.5	2.7	1.6
CI-898 + CTX	6.1	>3.1	>4.6
CI-898 + MTX	Toxic	1.4	NA[c]

[a] Logarithm of net tumor cell kill by the optimum combination regimen minus logarithm of net tumor cell kill by the optimal dose of the most active single agent.

[b] Abbreviations as in tables 1 and 2.

[c] NA = not applicable.

DISCUSSION

These studies demonstrated a high degree of confirmed therapeutic synergy between trimetrexate and doxorubicin, 6-TG, and cytoxan against P388 leukemia. The combinations of trimetrexate with FUra, vincristine, and possibly cisplatin also produced enhanced tumor cell kill at tolerated doses, although the degree of synergy was less than that for the agents discussed above. The synergy observed with 6-TG was obtained in spite of synergistic host toxicity. Similar patterns of synergy have been observed for many of these agents in combination with methotrexate (*12–21*). The combination of trimetrexate with methotrexate produces enhanced host toxicity that precluded demonstration of true therapeutic synergy with the treatment regimens used.

Several mechanisms may come into play in the production of therapeutic synergy of drug combinations in cancer therapy. Among these are biochemical potentiation, lack of cross-resistance between agents in the combinations, nonoverlapping host toxicities enabling administration of more therapeutic drug with the same overall toxicity, and kinetic effects on cell cycle distribution within the tumor that recruit cells into more sensitive parts of the cell cycle. Although the biochemical, toxicologic, and cell cycle effects of trimetrexate have been reasonably well studied, little is known of the patterns of cross-resistance between trimetrexate and other nonantifolate agents.

Although the mechanisms for the enhanced tumor cell killing of the trimetrexate combination regimens studied here have not been determined, it seems likely that the synergy of the trimetrexate combinations with doxorubicin and cytoxan may be due in part to the apparent lack of overlapping host toxicities of these agents as judged by their relatively high CTI values. However, the observed synergy of the CI-898 and 6-TG combination regimen was obtained in spite of apparently synergistic host toxicity. In light of the relatively weak activity of 6-TG as a single agent against P388 leukemia, a rationale for the observed synergy between trimetrexate and 6-TG based upon a lack of cross-resistance between trimetrexate and 6-TG is less attractive

Table 4.—Combination toxicity index determinations for CI-898 in combination with various clinically active anticancer drugs[a]

	Test 1		Test 2		
Combination[b]	Method 1	Method 2	Method 1	Method 2	Mean
CI-898 + DOX	2.0	NC[c]	1.7	NC	1.8
CI-898 + FUra	1.3	1.0	0.7–1.1	1.0	1.0
CI-898 + VCR	1.7	NC	1.1	NC	1.4
CI-898 + 6-TG	0.7	NC	0.5	NC	0.6
CI-898 + DDP	1.1	1.0	0.7	NC	1.0
CI-898 + CTX	1.6	NC	2.0	NC	1.8
CI-898 + MTX	<0.7	NC	0.4	NC	<0.5

[a] Method 1, CTI calculated on the basis of optimal response. Method 2, CTI calculated on the basis of estimated maximum tolerated dose.

[b] Abbreviations as in tables 1 and 2.

[c] NC = not calculated.

than one based on possible biochemical potentiation. The additive cell kill produced by trimetrexate and 6-TG as single agents would be insufficient to reduce the tumor burden to levels expected to select for trimetrexate-resistant cells. Inhibition of de novo synthesis of DNA precursors by CI-898 with subsequently enhanced incorporation of 6-TG into DNA might provide enhanced cell killing, however.

A lack of cross-resistance as well as enhanced cell killing due to a sequential blockade of de novo DNA precursor synthesis (inhibition of dihydrofolate reductase by trimetrexate and inhibition of thymidylate synthetase by FUra) are both possible mechanisms of enhanced cell killing by the trimetrexate–FUra combination.

Trimetrexate was selected for clinical trial on the basis of its superior spectrum of anticancer activity in preclinical models and lack of cross-resistance with methotrexate in transport deficient cell lines (*2,3,5,6,8–10*). Phase I results indicated that CI-898 was well tolerated. Responses were observed in colorectal, mammary, and lung cancer patients, among others, suggesting that trimetrexate may prove to be a clinically useful anticancer drug in future phase II and III trials. Most clinical cancer therapy relies on the use of multidrug combinations for reasons already discussed. The results presented here suggest that trials of trimetrexate in combination with some of the most active anticancer agents in the clinic are warranted. However, the degree of synergy observed for combinations of anticancer drugs is highly dependent on a variety of factors including relative dose intensities and sequence and schedule of administration (*21,22*). An examination of the effect of these factors will be the subject of a future report.

REFERENCES

(*1*) Elslager EF, Johnson JL, Werbel LM: Folate antagonists 20. Synthesis, antitumor, and antimalarial properties of trimetrexate and related 6-[(phenylamino)methyl]-2,4-quinazolinediamines. J Med Chem 26:1753–1760, 1983.

(*2*) O'Dwyer PJ, Shoemaker DD, Plowman J, et al: Trimetrexate: A new antifol entering clinical trials. Invest New Drugs 3:71–75, 1985.

(*3*) Jackson RC, Fry DD, Boritzki TJ, et al: Biochemical pharmacology of the lipophilic antifolate, trimetrexate. Adv Enzyme Regul 22:187–206, 1984.

(*4*) Hook KE, Nelson JM, Roberts BJ, et al: Cell cycle effects of trimetrexate (CI-898). Cancer Chemother Pharmacol 16:116–120, 1986.

(*5*) McCormack JJ, Heusner JJ, Mathews LA: Preclinical studies with the 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)-methyl]quinazoline (NSC 249008), a nonclassical antifolate. Proc Am Assoc Cancer Res 21:294, 1980.

(*6*) Bertino JR, Sawicki WL, Moroson BA, et al: 2,4-Diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)-methyl]-quinazoline (TMQ), a potent non-classical folate antagonist inhibitor. I. Effect on dihydrofolate reductase and growth of rodent tumors in vitro and in vivo. Biochem Pharmacol 28:1983–1987, 1979.

(*7*) Broome MG, Johnson RK, Evans SF, et al: Leucovorin reversal studies with TMQ and a triazine antifol in comparison with methotrexate. Proc Am Assoc Cancer Res 23:178, 1982.

(*8*) Kamen BA, Eibl B, Cashmore AR, et al: Efficacy and transport of a new lipid soluble antifol, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]-quinazoline (TMQ:JB-11) in methotrexate-resistant cells. Proc Am Assoc Cancer Res 22:26, 1981.

(*9*) Ohnoshi T, Ohnuma T, Takahashi I, et al: Establishment of methotrexate-resistant human acute lymphoblastic leukemia cells in culture and effects of folate antagonists. Cancer Res 42:1655–1660, 1982.

(*10*) Diddens H, Niethammer D, Jackson RC: Patterns of cross-resistance to the antifolate drugs trimetrexate, metoprine, homofolate, and CB3717 in human lymphoma and osteosarcoma cells resistant to methotrexate. Cancer Res 43:5286–5292, 1983.

(*11*) Weir EC, Cashmore AR, Dreyer RN, et al: Pharmacology and toxicity of the "non-classical" 2,4-diamino-quinazoline folate antagonist, trimetrexate, in normal dogs. Cancer Res 42:1699–1702, 1982.

(*12*) Leopold WR, Shillis JL, Mertus AE, et al: Anticancer activity of the structurally novel antibiotic CI-920 and its analogues. Cancer Res 44:1928–1932, 1984.

(*13*) Schabel FM Jr, Griswold DP Jr, Laster WR Jr, et al. Quantitative evaluation of anticancer agent activity in experimental animals. Pharmacol Ther 1:411–435, 1977.

(*14*) Geran RI, Greenberg NH, MacDonald MM, et al: Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother Rep 3:1–103, 1972.

(*15*) In vivo cancer models, 1976–1982, DHHS(PHS)84-2635. Washington, DC: U.S. Govt Print Off, 1984.

(*16*) Skipper HE: Combination Therapy: Some concepts and results. Cancer Chemother Rep 4(pt2):137–146, 1974.

(*17*) Goldin A, Johnson RK: Experimental tumor activity of Adriamycin (NSC 123127). Cancer Chemother Rep 6:137–145, 1975.

(*18*) Kline I, Venditti JM, Mead JAR, et al: The antileukemic effectiveness of 5-fluorouracil and methotrexate in the combination chemotherapy of advanced leukemia L1210 in mice. Cancer Res 26:848–852, 1966.

(*19*) Venditti JM, Goldin A: Drug synergism in antineoplastic chemotherapy. *In* Advances in Chemotherapy (Goldin A, Hawkins F, eds). New York: Academic Press, 1964, pp 397–495.

(*20*) Gale GR, Atkins LM, Meischen SJ, et al: Combination chemotherapy of L1210 leukemia with platinum compounds and cyclophosphamide plus other selected antineoplastic agents. J Natl Cancer Inst 57:1363–1366, 1976.

(*21*) Chello PL, Sirotnak FM, Dorick DM, et al: Schedule dependent synergism of methotrexate and vincristine against murine L1210 leukemia. Cancer Treat Rep 63: 1889–1894, 1979.

(*22*) Goldin A: Dosing and sequencing for antineoplastic synergism in combination chemotherapy. Cancer 54:1155–1159, 1984.

Trimetrexate: Clinical Development of a Nonclassical Antifolate

Peter J. O'Dwyer,[1,*] Robert J. DeLap,[2] Susan A. King,[1] A. J. Grillo-Lopez,[2]
Daniel F. Hoth,[1] and Brian Leyland-Jones[1]

ABSTRACT—**Trimetrexate is a 2,4-diaminoquinazoline inhibitor of dihydrofolate reductase (DHFR) which is cytotoxic in vitro and in vivo to several tumors resistant to methotrexate. It is more lipophilic than the parent antifolate, and is not transported by the reduced folate carrier. These features promise activity greater than that of methotrexate in the clinic; its inability to undergo polyglutamylation may also enhance the therapeutic index. In preclinical models, the activity of trimetrexate was highly schedule dependent, being superior on repeated dose schedules. Phase I studies have demonstrated that myelosuppression is the major toxic effect of trimetrexate on all schedules tested in man. Phase II studies will evaluate a 5-day schedule initially; trials in multiple tumor types and examination of the role of schedule are already under way.—NCI Monogr 5:105–109, 1987.**

Methotrexate remains the most active and frequently prescribed antimetabolite in clinical oncology. Studies of the determinants of its cytotoxicity and its cellular pharmacology allowed the rational modification of its structure to produce analogs with broader activity in preclinical studies. Trimetrexate is one of a series of lipophilic antifolates synthesized by Elslager et al. (*1,2*). Its structure differs from that of methotrexate in that: 1) it is a 2,4-diaminoquinazoline, and 2) it is "nonclassical," lacking a glutamate residue on the aromatic ring, which has instead 3 methoxy groups which prevent polyglutamylation (fig. 1). Trimetrexate retains potent inhibitory activity against DHFR (*3*), while exhibiting several additional characteristics which led to its further development.

Trimetrexate demonstrates activity equivalent to that of methotrexate against methotrexate-sensitive cell lines, while several murine and human methotrexate-resistant lines are sensitive to its cytotoxic action (*4,5*). Studies in murine models in vivo show that trimetrexate has activity superior to that of methotrexate in a number of solid tumors, notably B16 melanoma, colon 26, and colon 38 (*6*). The major reason for this superiority over the parent compound is believed to lie in the transport differences between them. Methotrexate enters cells by the reduced folate transporter, while trimetrexate is not a substrate for this mechanism. Kamen et al. have shown that trimetrexate transport occurs by a temperature-sensitive process which appears to represent a separate low-affinity carrier (*7*). This is consistent with the requirement for high concentrations of folinic acid (100 μM) to reverse the trimetrexate-induced inhibition of deoxyuridine into DNA (*8*). It is also supported by the observation that the human lines resistant to methotrexate but sensitive to trimetrexate are methotrexate resistant on the basis of a defect in transport; lines resistant on the basis of DHFR gene-amplification are partially or totally cross-resistant to trimetrexate (*9–11*).

The other major reason for interest in trimetrexate is its inability to be polyglutamylated intracellularly. While polyglutamylation is an important determinant of the cytotoxicity of methotrexate, it is also a factor in toxicity to normal tissues (*12*). Polyglutamylated methotrexate has affinity for DHFR and for several other enzymes of pyrimidine and purine biosynthesis greater than that of the parent monoglutamate and is retained intracellularly for prolonged periods (*13,14*). Since polyglutamylation of trimetrexate does not occur, it is anticipated that its major action would be restricted to inhibition of DHFR, and that removal of drug from the extracellular environment would restore DHFR activity promptly. Therefore, it is expected that the therapeutic index of trimetrexate may be superior to that of the parent compound in vivo.

PRECLINICAL ANTITUMOR STUDIES

The activity of trimetrexate in the NCI tumor panel is superior to that of methotrexate in the same systems (*6*). Three formulations of trimetrexate have been developed (acetate, glucuronate, and isethionate); all show similar degrees of preclinical activity (*6*). The preferred formulation for clinical use will depend on ease of manufacture. Phase I studies have been performed using the glucuronate salt, the formulation which is most suitable for intravenous administration. Studies of oral trimetrexate may use a different formulation as dictated by bioavailability. The activities of the glucuronate and isethionate salts given orally to tumor-bearing mice are equivalent to those of equal doses given iv (*6*).

Studies in P388 leukemia show the importance of schedule of administration in maximizing the antitumor activity of trimetrexate (fig. 2). On each schedule, superior results are obtained if the total dose per day is administered as 8 separate doses every 3 hours. This may reflect the very short half-life of trimetrexate in the mouse (45 to 50 min), and suggests that prolonged exposure to trimetrexate is an important factor (*15*). Further, schedules of frequent administration are uniformly more active than the single

---

ABBREVIATIONS: DHFR = dihydrofolate reductase; HPLC = high-pressure liquid chromatography; MTD = maximum tolerated dose.

---

[1] Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[2] Clinical Oncology Research Program, Pharmaceutical Research Division, Warner-Lambert/Parke-Davis, Ann Arbor, MI.

[*] *Reprint requests to:* Peter J. O'Dwyer, M.D., Fox Chase Cancer Center, Central and Shelmire Avenues, Philadelphia, PA 19111.

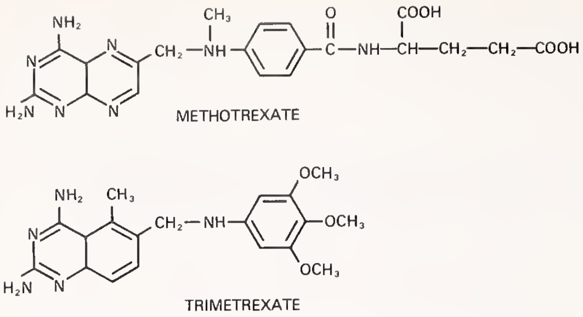


FIGURE 1.—Structure of trimetrexate and methotrexate.

dose schedule. Treatment on all schedules other than the single dose met the criterion of "activity" in this model. The most active schedule was associated with activity over an 8-fold dose range (*6*).

In addition to the striking results obtained with trimetrexate as a single agent, there is increasing preclinical evidence for its antitumor activity in combination regimens. Leopold et al., in this symposium, have summarized the synergistic interactions observed with several trimetrexate-containing combinations. Synergy was especially marked with cyclophosphamide, 6-thioguanine, and doxorubicin. Methotrexate displays synergy with many of the same agents. Studies in vitro and in vivo demonstrate consistently the importance of scheduling in maximizing the observed synergy of such combinations (*16*). In many cases, as with, for example, dichloromethotrexate and cisplatin, while the biochemical basis for the synergy is not understood, the scheduling studies performed in vivo provide a guide to more efficacious use of the drugs in combination (*16*). In others, such as the antifolate–5-fluorouracil combination, careful documentation of biochemical changes in the initial phase I studies may yield the most effective regimens for further testing (*17*).

PHASE I STUDIES OF TRIMETREXATE

Trimetrexate has been evaluated on a variety of schedules in adult and pediatric patients (table 1). Many of these studies have not yet been published and will not be discussed. However, the available information allows several conclusions to be drawn.

First, as with methotrexate, repeated administration (daily for 5 or 9 days) allows much less total drug to be administered than can be tolerated as a single dose. This observation probably relates to the cell-cycle specificity of antifolates.

Second, the dose-limiting toxicity on all schedules of administration is myelosuppression. This toxicity occurs early (days 8 to 10) and resolves rapidly (usually by day 14). In some studies, platelet depression was more severe than leukopenia; however, a clear relationship to schedule has not emerged and will have to await final results of all the studies. Mucositis is much less common than with methotrexate and has not been severe. On some schedules (most notably on the 24-hr infusion), dermatologic manifestations have been frequent (*22*). Apart from radiation recall, and cellulitis at the infusion site, 11 of 31 patients developed a generalized pruritic papular rash which went on to erythematous plaque formation. The rash characteristically began on the fourth day, resolved by the tenth day, did not require treatment, and did not recur on reexposure to the drug. Skin toxicity was less common and less severe on other schedules of administration. Minor elevations of liver enzymes in a small number of patients, and a single case of elevation of serum creatinine, were other reported effects of trimetrexate. The most striking feature of the myelosuppression was the variability of dose at which it appeared. In almost every study, 1 or 2 patients experienced grade 2 or 3 myelosuppression at the lowest doses, while subsequent escalations to 10 to 20 times that dose were tolerated by the majority of patients. Differences in hepatic and renal function did not explain this variability; it should be noted that normal renal function was required for eligibility for the initial phase I studies. However, it was clear that the patients who tolerated only very low doses of trimetrexate had received either radiation therapy to a significant proportion of the marrow-bearing skeleton, or extensive chemotherapy with myelotoxic drugs. This dependence of tolerable dose on extent of prior therapy was noted on all schedules of administration, and has led to recommended doses for phase II testing which are a function of prior treatment status at entry. On the 5-day bolus schedule, NCI-sponsored phase II studies will be conducted at 8 mg/m^2/d for 5 days for patients with previous chemotherapy or radiotherapy, while previously untreated patients will receive 12 mg/m^2/d for 5 days. Based on the rapid recovery of blood counts, cycles will be repeated every 21 days.

The phase I studies also indicate that dose modifications should depend on the response to the initial course and should occur in large steps. For example, among patients experiencing grade 3 or 4 toxicity to a given dose, reduction of 2 levels was often required to yield tolerable toxicity. Therefore, the appearance of this degree of myelosuppression to the initial course will require a 50% dose reduction. On the other hand, those who experience little toxicity to an initial dose should be escalated to a level at which a biological effect is noted. Increasing doses by 25% in such patients is expected to be safe. The phase I studies do not allow definitive conclusions to be made concerning cumulative toxicity following several courses of trimetrexate. Such observations will be an important goal of phase II trials.

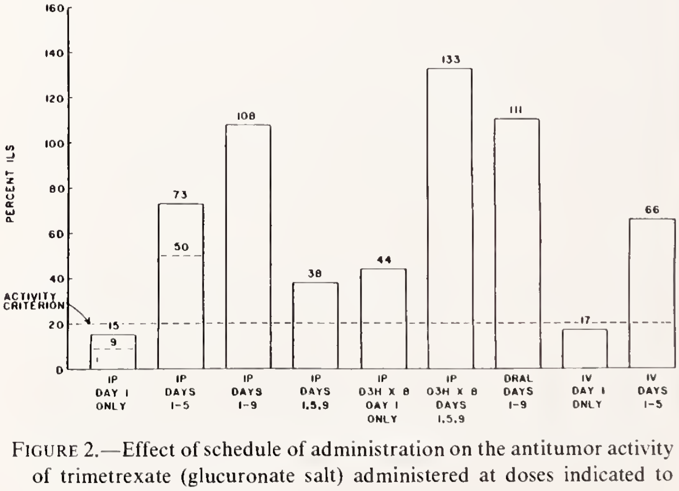


FIGURE 2.—Effect of schedule of administration on the antitumor activity of trimetrexate (glucuronate salt) administered at doses indicated to mice bearing P388 leukemia.

TABLE 1.—Phase I studies of trimetrexate

Schedule	Institution	MTD, mg/m^2	Dose-limiting toxicity	Reference
Adults				
d×1 bolus	Johns Hopkins	220	Myelosuppression	(*18*)
	Yale	200	Myelosuppression[a]	
	MD Anderson	200	Myelosuppression	(*19*)
24 hr-infusion	National Cancer Institute	200	Myelosuppression[b]	
d×5 bolus	Vermont	NA[c]	Myelosuppression	(*20*)
d×9 bolus	National Cancer Institute, Canada	NA	Myelosuppression[d]	
weekly×3	Memorial	110	Myelosuppression	(*21*)
Pediatrics				
d×5 bolus	Pediatric Oncology Group	NA	NA	
weekly×3	National Cancer Institute	NA	NA	

[a] Bertino JR, personal communication.
[b] Curt G, personal communication.
[c] NA = not available.
[d] Jolivet J, personal communication.

Finally, it is encouraging to note that responses to trimetrexate have been noted in all of the phase I studies. Among the tumors in which at least one partial response has been noted are colon, lung, head and neck, melanoma, and breast. Minor responses in heavily treated patients have been noted in many other tumor types. These results provide a strong stimulus to the further clinical development of trimetrexate.

PHARMACOLOGY IN MAN

The pharmacology of trimetrexate was studied on each of the phase I clinical trials (table 2). Studies of oral trimetrexate are in progress, but bioavailability data are not yet available. A study in the monkey (*23*), however, appears to confirm the data from murine studies in that bioavailability was 51%.

Plasma disappearance of trimetrexate in adults was described by a 2- or 3-compartment open model with a terminal half-life of approximately 12 hours (range 8.9 to 17.6). The half-life in children was shorter (6 hr). Total body clearance was consistent across studies (30 to 36 ml/min/m^2) and was considerably less than creatinine clearance. Urinary recovery was variable. When measured by high-pressure liquid chromatography (HPLC), it was generally below 20%, but by the DHFR inhibition assay, values were usually in excess of 50%. These data are consistent with metabolism of trimetrexate to a molecule which retains DHFR-inhibitory properties. Candidates for such a metabolite have been identified by Heusner et al.; loss of one or more methoxy groups occurs in vivo (*24*). The extent of hepatic metabolism of trimetrexate has not yet been determined. Many of the studies in progress also propose to evaluate the time course of DHFR inhibition fol-

TABLE 2.—Pharmacokinetics of trimetrexate

Institution (schedule)	Method	No.	Terminal half-life, hr	Total body clearance, ml/min/m^2	Vd_{ss}[a] liter/m^2	Urinary recovery, %	Reference
Johns Hopkins (d×1 bolus)	DHFR Inhibition	21	8.9	36.9	26	36 ± 22[b] (over 48 hr)	(*18*)
Yale (d×1 bolus)	DHFR Inhibition	16	11.5	NA[c]	NA	71–100[d]	
MD Anderson (d×1 bolus)	DHFR Inhibition	7	16	NA	NA	50 (over 24 hr)	(*19*)
NCI (adults) (24-hr CI)	DHFR Inhibition	12	10.8	NA	NA	56 ± 7[b,e] (over 24 hr)	
Memorial (weekly)	DHFR Inhibition	3	15.4	17	19.5	NA	(*21*)
Vermont (d×5 bolus)	HPLC	5	12	30	NA	9–78	(*20*)
NCI (pediatrics) (weekly)	HPLC	10	6.6	36.7	12.1	13.6[f]	

[a] Vd_{ss} = volume of distribution at steady state.
[b] Mean ± SE.
[c] NA = not available.
[d] Bertino JR, personal communication.
[e] Curt G, personal communication.
[f] Poplack D, personal communication.

lowing trimetrexate administration (*21*). Such data will be of value in designing combination regimens incorporating trimetrexate.

It is clear, however, from these data that the prolonged half-life of trimetrexate renders superfluous the continuous infusion schedule. The similarity of the maximum tolerated doses (MTD) of bolus and 24-hour infusion schedules confirms this conclusion.

As patients with abnormal renal function begin to be evaluated, it should be possible to derive criteria for dose-selection in a manner analogous to that achieved for carboplatin (CBDCA) (*25*). Individualization of patient dose may reduce some of the variability noted in the phase I studies.

FURTHER CLINICAL DEVELOPMENT OF TRIMETREXATE

For purposes of clinical development, new anticancer drugs may be divided into: 1) those with novel structures, and 2) analogs of active drugs already established in the clinic. For the former group, one hopes to cast a wide net in phase II studies and to examine a sufficient number of patients with as many tumor types as possible. Further testing would not ordinarily be undertaken until activity had been reproducibly shown. With an analog, however, one can target studies at the earliest stages to discover as rapidly as possible if the analog is superior to the parent compound. We have summarized elsewhere in this symposium the sequence of clinical studies that will be followed to address the merits of trimetrexate relative to methotrexate in the clinic.

In addition to these studies, trimetrexate offers the opportunity to study a question of considerable importance in the use of many cytotoxic drugs. In common with many antimetabolites, the antitumor activity of trimetrexate is highly schedule dependent (fig. 2). However, these differences in therapeutic index remain almost unexplored in the clinic, with the exception of studies of doxorubicin (*26*) and FUra (*27*). Both of these studies were important in that significant advantages to the experimental arm were demonstrated. Weekly doxorubicin was less toxic and as effective as a single dose every 3 weeks. FUra by infusion was more active than an intermittent schedule at equitoxic doses.

Trimetrexate is particularly suitable for such a test of schedule: In the preclinical models, it is highly schedule dependent. It exhibits a steep dose–toxicity curve in animals (*6*); it should therefore be possible to give trimetrexate in such a way as to achieve a reproducible biological effect. Trimetrexate has a well-defined mechanism of action; hence its effects can be followed in sequential biological samples. Finally, the toxic effects of trimetrexate are reversible with leucovorin, which may contribute to the safety of such a study.

Phase I studies of trimetrexate have defined MTD on several schedules. It is proposed to compare 2 of these schedules by the use of trimetrexate as a single agent. The schedules to be used will be at extremes: for example, daily for 5 days vs. single dose. The tumor type in which the question may be tested most suitably is head and neck cancer, a disease in which single agent antifolate therapy is currently standard across most of the country. The results of such a study would be important in first indicating the most appropriate schedule of trimetrexate administration, and second in validating the importance of scheduling studies in the mouse model. Such studies often determine how a drug will be developed in the clinic. However, their validity has never been established. For the reasons stated above, trimetrexate is an excellent candidate for such a test.

CONCLUSIONS

Trimetrexate is undergoing extensive clinical evaluation as a nonclassical antifolate. Phase I and phase II studies under the sponsorship of the National Cancer Institute and the Warner-Lambert Company are in progress. Comparison with methotrexate in methotrexate-sensitive tumors and broad evaluation in methotrexate-resistant diseases are planned. Clinical studies will also address the influence of schedule on the activity of trimetrexate in selected diseases.

REFERENCES

(*1*) Elslager EF, Davoll J: Synthesis of fused pyrimidines as folate antagonists. *In* Lectures in Heterocyclic Chemistry. (Castle RN, Townsend LB, eds) vol 2. Orem, Utah: Hetero Corp., 1974, pp 97–133.

(*2*) Elslager EF, Johnson JL, Werbel LM: Folate antagonists 20. Synthesis, antitumor, and antimalarial properties of trimetrexate and related 6-[(phenylamino)-methyl]-2,4-quinazoline-diamines. J Med Chem 26:1753–1760, 1983.

(*3*) Richter WE, McCormack JJ: Inhibition of mammalian dihydrofolate reductase by selected 2,4-diaminoquinazolines and related compounds. J Med Chem 17:943–947, 1974.

(*4*) Bertino JR, Sawicki WL: Potent inhibitory activity of trimethoxyquine (TMQ), a “nonclassical” 2,4-diamino-quinazoline, on mammalian DNA synthesis. Proc Am Assoc Cancer Res, ASCO 18:168, 1977.

(*5*) Bertino JR, Sawicki WL, Moroson BA, et al: 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]-quinazoline (TMQ), a potent nonclassical folate antogonist inhibitor. I. Effect on dihydrofolate reductase and growth of rodent tumors in vitro and in vivo. Biochem Pharmacol 28:1983–1987, 1979.

(*6*) O’Dwyer PJ, Shoemaker DD, Plowman J, et al: Trimetrexate: A new antifolate entering clinical trials. Invest New Drugs 3:71–75, 1985.

(*7*) Kamen BA, Eibl B, Cashmore A, et al: Uptake and efficacy of trimetrexate (TMQ, 2,4-diamino-5-methyl-6-[(trimethoxyanilino)methyl] quinazoline), a nonclassical antifolate, in MTX-resistant leukemia cells in vitro. Biochem Pharmacol 33:1697–1699, 1984.

(*8*) Broome MG, Johnson RK, Evans SF, et al: Leucovorin reversal studies with TMQ and a triazinate antifol in comparison with methotrexate. Proc Am Assoc Cancer Res 23:178, 1982.

(*9*) Ohnoshi T, Ohnuma T, Takahashi I, et al: Establishment of methotrexate-resistant human acute lymphoblastic leukemia cells in culture and effects of folate antagonists. Cancer Res 42:1655–1660, 1982.

(*10*) Diddens H, Niethammer D, Jackson RC: Patterns of cross-resistance to the antifolate drugs trimetrexate, metoprine, homofolate, and CB 3717 in human lymphoma and osteosarcoma cells resistant to methotrexate. Cancer Res 43:5286–5292, 1983.

(*11*) Jackson RC, Fry DW, Boritzki TJ, et al: Biochemical pharmacology of the lipophilic antifolate trimetrexate. Adv Enzyme Regul 22:187–206, 1984.

(*12*) Baugh CM, Krumdieck CL, Nair MG: Polygammaglutamyl metabolites of methotrexate. Biochem Biophys Res Commun 52:27–34, 1973.

(*13*) Jolivet J, Schilsky RL, Bailey BD, et al: Synthesis, retention, and biological activity of methotrexate in cultured human breast cancer cells. J Clin Invest 70:351–360, 1982.

(*14*) Jolivet J, Chabner BA: Intracellular pharmacokinetics of methotrexate polyglutamates in human breast cancer cells: selective retention and less dissociable binding of 4-NH_2-10-CH_3-PteGlu$_4$ and $_5$ to dihydrofolate reductase. J Clin Invest 72:773–778, 1983.

(*15*) Heusner JJ, McCormack JJ: Enzymatic assays for 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl] quinazoline, a promising new "nonclassical" antifolate. J Pharm Sci 70:827–828, 1981.

(*16*) Sobrero AF, Bertino JR: Sequence-dependent synergism between dichloromethotrexate and cisplatin in a human colon carcinoma cell line. Cancer Treat Rep 62:279–283, 1985.

(*17*) Leyland-Jones B, O'Dwyer P: Biochemical modulation: application of laboratory models to the clinic. Cancer Treat Rep 70:219–229, 1986.

(*18*) Donehower RC, Graham ML, Thompson GE, et al: Phase I and pharmacokinetic study of trimetrexate (TMTX) in patients with advanced cancer. Proc ASCO 4:32, 1985.

(*19*) Legha S, Tenney D, Ho DH, et al: Phase I clinical and pharmacology study of trimetrexate (TMQ). Proc ASCO 4:48, 1985.

(*20*) Stewart JA, McCormack JJ, Tong W, et al: Phase I study of trimetrexate. Proc Am Assoc Cancer Res 26:159, 1985.

(*21*) Fanucchi M, Fleisher M, Vidal P, et al: Phase I and pharmacologic study of trimetrexate (TMTX). Proc Am Assoc Cancer Res 26:179, 1985.

(*22*) Weiss RB, James WD, Major WB, et al. Skin reactions induced by trimetrexate, an analog of methotrexate. Invest New Drugs 4:159–163, 1986.

(*23*) Balis FM, Lester CM, Poplack DG: Pharmacokinetics of trimetrexate (NSC 352122) in monkeys. Cancer Res 46:169–174, 1986.

(*24*) Heusner JJ, Tong WP, McCormack JJ, et al: Disposition of the non-classical folate antagonist 2,4-diamino-5-methyl-6-(3,4,5-trimethoxyanilino) methylquinazoline (TMQ; trimetrexate) in mice. Proc Am Assoc Cancer Res 26:234, 1985.

(*25*) Egorin MJ, Van Echo DA, Olman EA, et al: Prospective validation of a pharmacologically based dosing scheme for the *cis*-diamminedichloroplatinum(II) analogue diamminecyclobutane-dicarboxylatoplatinum. Cancer Res 45:6502–6506, 1985.

(*26*) Valdivieso M, Burgess MA, Ewer MS, et al: Increased therapeutic index of weekly doxorubicin in the therapy of non-small cell lung cancer: A prospective randomized study. J Clin Oncol 2:207–214, 1984.

(*27*) Ansfield F, Klotz J, Nealon T, et al: A phase III study comparing the clinical utility of four regimens of 5-fluorouracil: A preliminary report. Cancer 39:34–40, 1977.

Preclinical Biochemical Pharmacology and Toxicology of Piritrexim, a Lipophilic Inhibitor of Dihydrofolate Reductase

C. W. Sigel, A. W. Macklin, J. L. Woolley, Jr., N. W. Johnson, M. A. Collier, M. R. Blum, N. J. Clendeninn, B. J. M. Everitt, G. Grebe, A. Mackars, R. G. Foss, D. S. Duch, S. W. Bowers, and C. A. Nichol [1,2]

ABSTRACT—Piritrexim (PTX), 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-*d*]pyrimidine, formerly called BW 301U, is a potent small-molecule inhibitor of dihydrofolate reductase (DHFR) that enters cells rapidly by passive diffusion and thus does not depend upon the transport-mediated uptake that can limit cell entry of methotrexate (MTX). PTX is as active as MTX in inhibiting DHFR and mammalian cell growth. In vivo, PTX is active against Walker 256, L1210, P388, Sarcoma 180, and Ehrlich ascites tumors. After iv administration of [^{14}C]PTX to rats, the elimination profile of intact drug from plasma was first order with a half-life (t½) of 38 minutes. PTX penetrates extensively into tissues and its tissue:plasma concentration ratios are generally 10-fold higher than those reported for MTX. When administered systemically, PTX inhibits the DHFR-dependent conversion of sepiapterin or 7,8-dihydrobiopterin (BH_2) to tetrahydrobiopterin (BH_4), demonstrating that PTX enters brain at pharmacologically relevant concentrations. Pharmacokinetic studies in the dog indicated a mean plasma t½ (after iv dose) of 2.15 hours, total body clearance of 0.625 liters/hr/kg and steady-state volume of distribution of 1.82 liters/kg; the absolute bioavailability was 0.64. Toxicologic studies were conducted in rats and dogs that received daily doses for 1, 5, or 90 days. In dogs, oral doses of 480 (single dose), 25 (5 daily doses), and 2.5 mg/kg (90 daily doses) were lethal, whereas 240 (single dose), 2.5 (5 daily doses), and 0.5 mg/kg (90 daily doses) produced reversible alterations in clinical toxicity and histopathologic parameters. The lethal toxicity of PTX in dogs given 25 mg/kg/day for 5 days is prevented by oral calcium leucovorin rescue with either 0.75 or 3.0 mg/kg every hour for 4 hours on any of the 5 treatment days. The general pharmacologic profile indicates that PTX should be free of CNS, cardiovascular, and respiratory side effects at clinically useful doses.—NCI Monogr 5:111-120, 1987.

In recent years considerable attention has been given to the development of anticancer agents that are potent lipophilic inhibitors of DHFR. This research stems from clinical experience in the use of MTX with a limited spectrum of responsive tumors and from the development of resistance which results from restricted transport into target cells due to poor membrane permeability and the need for carrier-mediated active transport. The small-molecule antifolate compounds PTX (2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-D]pyrimidine; fig. 1), formerly called BW 301U (*1*), and trimetrexate (2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanalino)methyl]quinazoline) (*2*) currently under clinical investigation enter cells by passive diffusion offering the prospect for treatment of tumors unresponsive to MTX due to resistance arising from a transport mechanism or overproduction of DHFR as a result of gene amplification (*3–5*). The contrasting physical-chemical properties of MTX and the lipophilic antifols also lead to important differences in disposition and metabolism (*6,7*). PTX penetrates into cancer cells more rapidly (*4,8*) and into tissue compartments more extensively than MTX and is not metabolized to polyglutamates, characteristics that could result in profound differences in mechanisms of antitumor activity as well as in toxicity.

At The Wellcome Research Laboratories, the study of the lipophilic diaminopyrimidine inhibitors of DHFR (*9*) as antineoplastic agents began with pyrimethamine and metoprine (2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine) (*10,11*). Metoprine had antitumor activity in man, but its potential was limited by its long t½ (approximately 10 days in man) and its marginal efficacy at tolerated doses (*12–17*). Subsequently, metoprine was found to be a potent inhibitor of histamine *N*-methyltransferase, and it appeared likely that some of the non-folate-related toxicity was due to elevation of histamine concentrations (*8*). In a search for anticancer compounds with more selective toxicity, PTX was found to possess activity comparable to MTX as an inhibitor of DHFR, but it had much less effect than metoprine on the catabolism of histamine (*8*). These favorable properties prompted the extensive preclinical study of the biochemical pharmacology and toxicology of piritrexim.

BIOCHEMISTRY AND MECHANISM OF ACTION

When tested against a DHFR from human chronic granulocytic leukemia, piritrexim had activity [inhibitory concentration (IC_{50}) = 5 n*M*] comparable to that of MTX while being 18-fold less active than metoprine as an inhibitor of histamine methylation. PTX had no effect on histamine concentrations in rat brain at doses that caused a 2.5- to 5-fold increase in histamine after the administration of metoprine (*18*).

PTX appears to derive its antifolate effect by selective action on DHFR since at concentrations of 25 to 50 μM it

---

ABBREVIATIONS: BA_2 = 7,8-dihydrobiopterin (sepiapterin); BH_4 = tetrahydrobiopterin; DHFR = dihydrofolate reductase; LSC = liquid scintillation counting; MTX = methotrexate; PTX = piritrexim; t½ = half-life; TLC = thin-layer chromatography.

[1] The Wellcome Research Laboratories, Research Triangle Park, NC.

[2] The authors wish to acknowledge the technical assistance provided by M. Beaman, M. Catching, C. Goss, C. Hancock, R. Harper, A. Hatley, P. Jeffries, D. Kinney, K. Kusumi, B. Rohman, and E. M. Smith.

* *Reprint requests to:* C. W. Sigel, Ph.D., The Wellcome Research Laboratories, 3030 Cornwallis Road, Research Triangle Park, NC 27709.

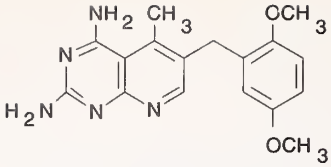


FIGURE 1.—Structure of PTX.

does not inhibit the tetrahydrofolate-requiring enzymes serine transhydroxymethylase, N^5-formyltetrahydrofolate cyclodehydrase, N^5,N^{10}-methylenetetrahydrofolate dehydrogenase, N^5,N^{10}-methylenetetrahydrofolate cyclohydrolase, N^{10}-formyltetrahydrofolate synthetase and thymidylate synthetase (*8*).

Transport studies in cell culture (fig. 2) showed that PTX enters cells [sarcoma 180 (*8*) and the human lymphoblastoid cell line WIL-2 (*4*)] more rapidly than MTX by a temperature-independent mechanism. In cytotoxicity studies in cell culture with Walker 256, sarcoma 180, and L1210 cells, PTX was at least as potent as MTX and considerably more active than metoprine in preventing cell growth (*8*).

The activity of PTX against MTX-resistant cell lines was evaluated by several investigators. Scanlon and coworkers (*3*) tested PTX against the MTX-resistant human acute lymphoblastic leukemia cell lines, MOLT-3/MTX_t, which is transport resistant to MTX, and MOLT-3/MTX_{te}, which is transport resistant to MTX and has increased DHFR activity. Collateral sensitivity was observed for PTX to both MOLT-3/MTX lines; PTX had ID_{50} concentrations required to inhibit 50% of growth) of 10^{-8} for the resistant lines and 10^{-7} for the parent line. For concentrations of PTX in the range of 3×10^{-9} to 3×10^{-7} *M*, equimolar concentrations of calcium leucovorin reversed the toxic effects of PTX to the parent line, but only partially reversed the toxicity to the MOLT-3/MTX_t line and did not reverse at all the toxicity to the MOLT-3/MTX_{te} line.

In similar studies with a DHFR-gene-amplified cell line of mouse 3T6 cells, Sedwick et al. found that PTX was approximately 1000 times more effective than MTX in inhibiting deoxyuridine incorporation into DNA. These cells are resistant to MTX because of overproduction of a DHFR which is 100 times more resistant to MTX than the enzyme from the parent cell (*4*).

Taylor et al. evaluated the activity of PTX against 3 MTX-resistant lines and the parent cells from which they were derived (*5*). Collateral sensitivity was observed in the CCRF-CEM/MTX R cells, a human T-cell line which is MTX resistant due to changes in transport; PTX was as effective in killing CCRF-CEM/MTX R as was MTX in killing the parent cells. In contrast PTX was more effective than MTX against the MTX-resistant lines, L5178Y/MTX and L1210/MTX, where MTX resistance is due to either DHFR overproduction or combined gene amplification and transport resistance, respectively, but it did not have collateral sensitivity.

Overall, the data from the studies with MTX-sensitive and -resistant cells indicate that PTX enters cells by a different mechanism than MTX or the reduced folate cofactors. In addition, the results suggest that PTX may be particularly effective when resistance is due primarily to a transport defect; however, resistance arising by gene amplification can also be overcome, to some degree, by the lipid-soluble antifols.

In the human tumor cloning assay, PTX compares favorably with MTX. PTX was tested over a concentration range of 0.05 to 10 μg/ml with continuous or 1-hour exposure times. The percentage of tumors responsive was not concentration-dependent when continuous exposure was used; for 1-hour exposures, 5 μg/ml was the optimal concentration (*19*). Activity (<50% cell survival after a 1-hour exposure to PTX at a concentration of 10 μg/ml) was observed in lung (3 of 16), ovarian (2 of 15), breast (1 of 14), and colon (2 of 7) cancers. Activity was observed also in carcinoma of unknown primary, lymphoma, myeloma, neuroblastoma, and melanoma.

Initial chemotherapy tests in animals were conducted using the Walker 256 carcinoma implanted sc in rats (*20*). This tumor is refractory to MTX, but it is highly responsive to PTX (fig. 3).

In other chemotherapy tests using several transplanted tumors in mice, PTX had significant antitumor activity (table 1) against P388 leukemia, sarcoma 180, and Ehrlich ascites carcinoma; it was marginally active against L1210 leukemia and B16 melanoma (*8*).

DISPOSITION AND PHARMACOKINETICS IN LABORATORY ANIMALS

Tissue Distribution and Pharmacokinetics in the Rat

Male Charles River Sprague-Dawley rats (205 to 265 g) were given [^{14}C]PTX hydrochloride (5 mg/kg) in 1.25 ml propylene glycol diluted in 5% dextrose (1:19) by bolus injection into the tail vein. Groups of 3 rats were killed by exsanguination after a sedative dose of sodium pentobarbital (ip) at 0.25, 0.5, 1, 3, 4, 8, 12, and 24 hours after the dose. In addition to blood and plasma, the following tissues were removed from rats killed at 0.25, 0.5, 1, 3, 8, and 24 hours, and stored at −20° C: brain, CSF, fat, heart, kidney, liver, lung, pancreas, muscle (quadriceps), skin-hair,

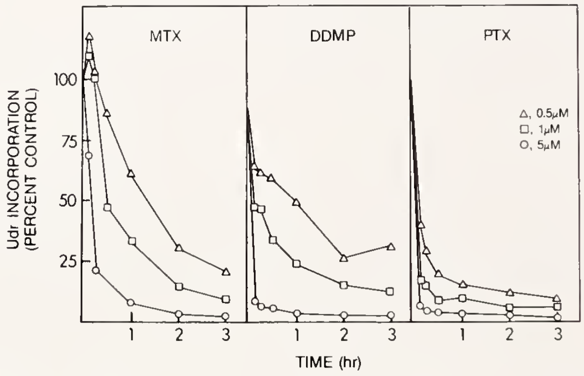


FIGURE 2.—Effect of antifolates on the inhibition of deoxyuridine incorporation by sarcoma 180 cells. Sarcoma 180 cells (2×10^5 cells) were seeded in 35-mm petri dishes and incorporation studies were carried out 48 hr later. The medium was replaced with 2 ml of folate- and serum-free Dulbecco modified Eagle medium (DMEM) containing PTX, metoprine (DDMP), or MTX at concentrations of 0.5 (△), 1 (□), or 5 (○) μ*M*. Incubation was for 10 sec to 2 hr after which the medium was removed and the cells washed with ice-cold folate- and serum-free DMEM. The monolayers were taken up in 2 ml of 0.1% SDS and sonicated for 20 sec, and 1 ml was counted in 15 ml Aquasol. Reprinted with permission from Cancer Res 42:3987–3994, 1982.

spleen, testes, stomach, small intestine, large intestine (stomach and intestine contents were removed before storage), adrenals, eyes, and bone (femur). Samples of blood, plasma, brain, kidney, liver and lung were taken from rats killed at 12 hours and only blood and plasma were taken from rats killed at 4 hours. The 3 animals killed at 24 hours were housed in individual glass metabolism cages designed for complete separation of urine and feces.

Total radioactivity in tissues was determined by combustion and liquid scintillation counting (LSC). Intact drug was measured by LSC after separation from metabolites by thin-layer chromatography (TLC).

[^{14}C]PTX was eliminated rapidly and linearly during the first 3 hours after iv administration, but the overall elimination profile appeared at least biphasic with a prolonged terminal elimination phase. A similar multicompartment pharmacokinetic profile for MTX in the rat has been reported (*21*).

During the first hour after the dose, high concentrations of radiolabeled material were found in the kidney and intestine; liver, stomach, adrenals, lung, and plasma also had significant concentrations (table 2). Total radioactivity was depleted rapidly from the tissues and generally paralleled the plasma profile. As observed for plasma, the concentrations of radioactivity in tissues declined very slowly between 3 and 24 hours (fig. 4). Examination of the excreta, however, indicated that during the 24 hours after the iv dose the male rat excreted an average of 23.4% of the dose in the urine and 74.8% in the feces.

Intact drug in the tissues was measured using a specific TLC method (*22*). In the first 3 hours after the dose, intact drug accounted for an average of 62%, 54%, 21%, and 2% of the radioactivity in brain, lung, kidney, and liver, respectively. The concentrations of PTX in lung are of considerable interest because MTX penetrates into lung poorly as evidenced by the steady-state MTX concentrations in rabbit lung (*23*) which were only 0.5% of the corresponding plasma concentrations (table 3).

An observation relevant to the study of PTX as a potential antipsoriatic agent is that the drug appears to have an

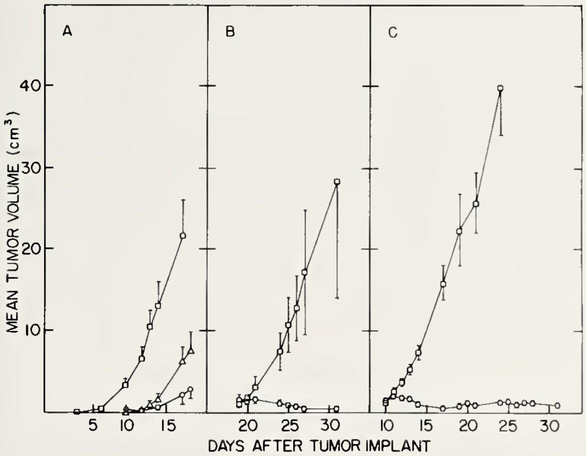


FIGURE 3.—Antitumor effects of PTX. Panel A—Fragments of Walker carcinoma 256 (1 mm^3) were implanted sc in the right flank of rats. Drug treatment was started on the third day after tumor implantation and continued for 5 successive days. (□) Untreated tumor controls; (△) 25 mg/kg daily; (○) 15 mg/kg twice daily. Each point represents the mean tumor volume for 20 rats. Panel B—Tumor-bearing rats which had been treated with PTX were selected from A and divided into 2 groups of 5 animals each. One group received no further treatment, while the second received 30 mg/kg daily on days 19 to 21 and 15 mg/kg twice daily on days 24 to 26. (□) Untreated tumor controls (no further treatment); (○) PTX. Panel C—Tumors were implanted sc and treatment was delayed until 10 days after tumor implantation. (□) Untreated tumor controls; (○) PTX, 30 mg/kg daily on days 10 to 13, 20, and 24, and 50 mg/kg on day 28. Each point represents the mean tumor volume of 22 animals. PTX was administered ip and untreated controls received physiological saline instead of drug. Mean tumor volumes were calculated using only those animals having measurable tumors. Reprinted with permission from Cancer Res 42:3987–3994, 1982.

TABLE 1.—Antitumor activity of PTX against murine tumors

Tumor	Dose, mg/kg	Dose schedule, days	% of increase in median lifespan
P388	67	1,5,9	75
	100	1,5,9	80
	150	1,5,9	105
	225	1,5,9	90
L1210	10	1–9	11
	15	1–9	17
	20	1–9	22
	50	1–4,7,8	56
	50	1–9	51
	100	1,2,4	33
Sarcoma 180	25	1–4,7–10	136
	50	1,4,7–10	164
	100	1,2,4,7	145
	200	1,4	209
Ehrlich	25	1–4,7,8,10	321
	50	1–4,7–9	67
	50	1–4,7,8,10	211
	100	1,2,4,8,10	116
	100	1,3,7,10	263

TABLE 2.—Concentrations of total radioactivity (μg/g [^{14}C]PTX equivalents) and intact PTX (μg/g) in rat tissues 1.0 hour after a single IV dose (5 mg/kg)

Tissue	Total radioactivity, mean ± SD	Intact drug, mean ± SD
Plasma	1.588 ± 0.768	0.667 ± 0.507
Blood	0.955 ± 0.407	ND[a]
Brain	0.075 ± 0.025	0.040 ± 0.007
Fat	0.270 ± 0.190	0.099 ± 0.103
Heart	0.789 ± 0.522	ND
Kidney	10.982 ± 6.264	1.142 ± 0.772
Liver	8.425 ± 1.752	0.164 ± 0.090
Lung	2.496 ± 1.888	0.895 ± 0.391
Pancreas	0.579 ± 0.118	0.289 ± 0.273
Muscle	0.514 ± 0.233	ND
Skin/hair	0.775 ± 0.211	0.421 ± 0.102
Spleen	1.013 ± 0.607	ND
Testes	0.312 ± 0.127	ND
Stomach	2.643 ± 1.588	ND
Small intestine	51.847 ± 39.243	6.791 ± 2.542
Large intestine	2.252 ± 0.596	1.557 ± 0.472
Adrenals	2.519 ± 1.184	ND
Eyes	0.354 ± 0.109	ND
Bone	0.473 ± 0.148	ND

[a] ND = not determined.

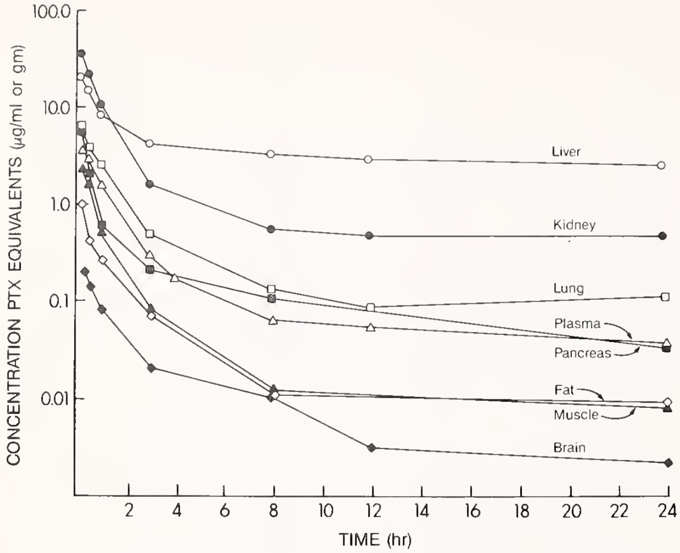


FIGURE 4.—Elimination of total radioactivity (μg/g or ml) of [^{14}C]PTX equivalents from plasma and various rat tissues after a single iv dose (5 mg/kg) of PTX.

affinity for skin. The elimination of PTX from skin does not parallel its elimination from plasma; during the first hour after the iv dose, concentrations in skin and plasma are comparable but at 8 hours after the dose, concentrations of intact PTX in skin are 16 times greater than those in plasma.

Of the 20 tissues analyzed, brain tissue consistently had the lowest concentrations of total radioactivity, reaching approximately 5% (10% of the intact PTX concentration) of the plasma concentration at each time point. These results were in marked contrast to those observed previously for metoprine which achieved concentrations in brain 10-fold higher than in plasma (*11*). While the PTX concentrations in brain were unexpectedly low, PTX still achieves brain-to-plasma concentration ratios about 10-fold greater than those reported for MTX in rabbit (*23*), and one might predict that PTX will achieve concentrations sufficient to inhibit DHFR activity in brain (table 3). The plasma concentrations observed in patients are in the range of 1 to 5 μg/ml, and therefore the corresponding concentrations in brain should be in the range of 0.15 to 0.75 *μM*.

Recent work of Duch and coworkers has provided a functional test for the in vivo determination of antifolate entry and efficacy in brain (*24*). Their model is based upon the observation that intraventricularly injected sepiapterin or BH_2 can serve as a precursor for the formation of BH_4 in brain by a pathway dependent upon DHFR for the conversion of BH_2 to BH_4 (*25*). Studies have shown that PTX (20 mg/kg ip) given 1 hour before the injection of BH_2 into brain completely inhibited the conversion for 90 minutes; a 5 mg/kg dose gave partial inhibition for up to 9 hours. Thus, while the entry of PTX into brain is greatly reduced from that of metoprine (*6*), it still may be useful for treatment of brain tumors and has a limited potential for CNS side effects (*24*).

In a separate study, the disposition of [^{14}C]PTX was studied in the rat following both iv and oral administration. To obtain serial blood samples from each rat, a jugular vein cannula was installed using a modification of the procedure of Upton (*26*). After iv doses of 5 and 10 mg/kg, the elimination profile of intact drug was approximately first order with an average t½ of 38 minutes and total body clearance of 5.1 liters/hr/kg. After oral doses of 5 and 10 mg/kg, the concentrations of total radioactivity in plasma were low, with maximum concentrations of only 0.08, 0.14, and 0.50 μg/ml, respectively, which are only 1% to 2% of those obtained after the same iv dose. There was virtually no intact drug in the plasma after the 10 mg/kg oral dose. The low drug concentrations in plasma after the oral dose apparently result from extensive first-pass metabolism as well as poor absorption; concentrations of total radioactivity in the liver 24 hours after the oral dose were approximately 40% of the concentrations found in the livers of animals treated with equivalent iv doses. A study on the major routes of metabolism of PTX by the rat is underway.

Pharmacokinetics in the Dog

Pharmacokinetic profiles of PTX isethionate in dogs were compared after iv infusion and oral administration to the same animals in a cross-over design study (*27*). Initially each of 3 male beagle dogs received by gavage a 10 mg/kg dose of PTX dissolved in 5% aqueous dextrose. Two weeks later an appropriate volume of PTX solution [1,2-propanediol:H_2O:5% dextrose (1:1:18)] was administered to the same dogs by iv infusion at a rate of 2.0 ml/minute. Drug concentrations were measured using a specific and sensitive high-pressure liquid chromatographic method (*27*). The plasma concentration-time data after iv treatment was well-fitted using the nonlinear least-squares regression program, NONLIN, in conjunction with a specific subroutine for a 2-compartment open-model with a zero-order input function (*28*). Model-independent pharmacokinetic

TABLE 3.—Comparison of drug tissue: plasma concentration ratios for MTX and PTX

Tissue	MTX, μg/ml or g[a]	MTX, T/P ratio[b]	PTX, μg/ml or g[c]	PTX, T/P ratio	TP_{PTX}/TP_{MTX}[d]
Plasma	156	1.0	0.67	1.0	—
Brain	0.95	0.006	0.04	0.06	10
Lung	7.35	0.05	0.89	1.32	26.4
Liver	0.78	0.005	0.16	0.24	48
Kidney	258	1.65	1.14	1.70	1.03
Large intestine	9.44	0.06	1.56	2.33	39

[a] MTX data from rabbits that received high-dose MTX (approximately 70 mg/kg) by infusion; steady-state concentrations were reported (*22*).

[b] Ratio of concentration in tissue to concentration in plasma.

[c] PTX data from rats, plasma and tissue concentrations of intact PTX at 1 hr after an iv dose (5 mg/kg).

[d] T/P ratio for PTX divided by T/P ratio for MTX.

parameters were also determined. Area under the plasma concentration-time curve was calculated by the trapezoidal rule. Because of the lack of an apparent distributional phase after oral dosing of PTX, the plasma concentration-time data were fit to a 1-compartment model with first-order absorption. After the iv dose, the mean terminal t½ was 2.15±0.49 hours. Total body clearance was 0.625±0.10 liter/hr/kg and the steady-state volume of distribution was 1.82±0.60 liter/kg. The mean pharmacokinetic parameters after iv and oral administration are shown in table 4. After oral administration, the mean peak concentration was 1.73±0.77 μg/ml occurring at 1.35±0.37 hour. Although there were interanimal differences in the PTX t½ after iv and oral dosing, the t½ within the same animal was quite consistent. The volume of distribution after oral dosing for individual dogs corresponded closely to the volume of distribution after iv infusion. The average absolute bioavailability of PTX was 0.64±0.047 as calculated by model-independent means and 0.64±0.025 calculated by a model-dependent method.

PHARMACOLOGY

The acute pharmacodynamic properties of PTX were evaluated in a range of pharmacological procedures to determine whether the compound had significant CNS, cardiovascular, autonomic, or other properties. Unless stated otherwise, PTX isethionate was administered as a solution or suspension in 10% Tween 80 in water in all in vivo studies.

General Pharmacology Over an Extended Dose Range in Rodents

The effects of PTX isethionate were studied following ip administration to groups ($N=4$) of non-fasted male mice (19 to 24 g) and Sprague-Dawley male rats (220 to 250 g) obtained from Charles River Laboratories.

PTX was well tolerated during the 4 hours following administration to both rats and mice. During this time there were no significant effects on respiration rate or behavioral reflexes (traction, prehensile, coordination, pinna, Haffner, righting, corneal and visual placing) in either species at doses up to 500 mg/kg. The only observable effect in rats or mice treated with PTX at 40 mg/kg was slight writhing. PTX was also well tolerated in mice at 100 mg/kg. In rats, this dose produced decreased abdominal muscle tone and piloerection during the 7 days following dosing and 1 animal died. More pronounced symptoms (blepharospasm, hypothermia, bloody nasal exudate, hind quarter elevation, bloody stool) and mortalities occurred in both rats and mice during the 7 days following treatment with doses of 250 to 500 mg/kg.

Tests for CNS Activities

In mice, PTX isethionate (70 mg/kg ip; 30 min pretreatment) failed to inhibit the convulsive seizures induced by pentylenetetrazole (100 mg/kg sc), the hind limb extensor component of the maximal seizures induced by electroshock, the tremors, extension of the front legs, and arching of the back induced by oxotremorine (0.5 mg/kg sc) and the blepharoptosis, sedation, and hypothermia induced by tetrabenazine (35 mg/kg ip). In addition, PTX isethionate (70 mg/kg ip; 15 min pretreatment) did not affect the stereotyped behavior and aggression induced in pairs of Long-Evans rats during the 60 minute period following the administration of apomorphine hydrochloride (5 mg/kg sc). Anorectic effects were detected in fasted mice (ICR; Blue Spruce Farms) during the 6 hours following the administration (ip) of PTX isethionate at 50 and 100 mg/kg but not at 25 mg/kg.

TABLE 4.—Mean pharmacokinetic parameters for PTX in the dog after oral and iv administration in a cross-over study

Parameter (iv dose)[a]	Mean ± SD	Parameter (oral dose)[b]	Mean ± SD
α, hr^{-1}	18.7±7.0	C_{max}, μg/ml	1.73±0.77
β, hr^{-1}	0.334±0.076	T_{max}, hr	1.35±0.37
t½β, hr	2.15±0.49	k_a, hr^{-1}	1.64±0.45
k_{10}, hr^{-1}	1.71±0.47	k_{el}, hr^{-1}	0.323±0.08
k_{12}, hr^{-1}	13.7±6.1	t½, hr	2.24±0.56
k_{21}, hr^{-1}	3.61±0.94	AUC, μg/ml/hr	7.75±1.41
AUC, μg/ml/hr	12.2±2.1	Cl, liters/hr/kg	0.98±0.16
Cl_{tot}, liters/hr/kg	0.625±0.10	Vd/F, liters/kg	3.25±1.23
Vd_{β}, liters/kg	1.97±0.66	Vd/liters/kg	2.07±0.75
Vd_{ss}, liters/kg	1.82±0.60		

[a] Data analyzed using a 2-compartment open model where: α = rapid disposition rate constant; β = slow disposition rate constant; k_{10} = elimination rate constant; k_{12} = distribution rate constant into peripheral compartment; k_{21} = distribution rate constant out of peripheral compartment; Cl_{tot} = total body clearance; Vd_{β} = volume of distribution beta; Vd_{ss} = volume of distribution, steady-state; AUC = area under the plasma concentration time curve from zero to infinity.

[b] Data analyzed using a 1-compartment model with first-order absorption where C_{max} = peak plasma concentration; T_{max} = time of peak plasma concentration; k_a = first order rate constant for absorption; k_{el} = first order rate constant for elimination, Vd = volume of distribution; Cl = total body clearance; F = extent of bioavailability.

Cardiovascular and Autonomic Studies

Local Anesthetic and Anti-arrhythmic Studies

A 5% suspension of PTX isethionate in water failed to show local anesthetic activity 5 to 30 minutes after topical application to the eyes of a guinea pig (*29*). In addition, PTX isethionate (70 mg/kg ip) afforded no protection against chloroform-induced ventricular fibrillation in mice and, at 10 μ*M* (30 min incubation), did not significantly affect the maximal following rate (*30*) of electrically-driven atrial preparations from the cat in vitro.

Direct Effects on Isolated Cardiac Muscle Preparations

Slight positive inotropic effects [up to 15±9% (mean±SE) increase in developed tension] were observed in electrically-driven cat atrial ($N=4$) and papillary ($N=5$) muscle preparations (*31*) after 30 minute incubations with PTX at 10 μ*M*.

PTX had weak negative chronotropic effects on spontaneously-beating guinea pig atria; the mean ($N=4$) resting rate of contractions was decreased 15±3% after a 15-minute incubation at 10 μ*M* and 66±19% at 30 μ*M*.

Direct Effects on Isolated Smooth Muscle Preparation

There were no direct effects on guinea pig ileum or rat stomach preparations incubated for 15 minutes with PTX at 10 μ*M;* rabbit aorta preparations were unaffected at 30 μ*M*. Preparations of guinea pig trachea with spontaneous tone relaxed after incubation with PTX, 1 to 30 μ*M;* at

30 μM the mean response was equivalent to 48% of the maximal relaxation induced by L-norepinephrine.

Effects of Peripheral Receptors and Responses to Autacoids

On rabbit aortic preparations PTX (30 μM; 15-min pre-incubation) produced only minor displacements (up to 2.1-fold) of cumulative concentration–response curves to L-norepinephrine and angiotensin II. Maximal responses of ileum to acetylcholine and bradykinin and of rat stomach to 5-hydroxytryptamine were inhibited (10 to 46%) by PTX at 30 μM (15-min pre-incubation). Submaximal responses of rat stomach to arachidonic acid were also inhibited by PTX at this concentration.

Only minor displacements (up to 1.7-fold) of the mean ($N=3$) concentration–response curves to the positive chronotropic effects of isoproterenol and histamine on spontaneously beating guinea pig atria occurred after PTX, 10 μM. In addition, relaxations of guinea pig trachea to L-norepinephrine were unaffected by PTX at 30 μM.

In Vivo Studies in the Rat and Dog

Relatively minor increases in systolic blood pressure (up to 16 ± 15%) and heart rate (up to 9 ± 3%) occurred in conscious normotensive rats ($N=4$) during the 4 hours following the administration of PTX isethionate at 25 mg/kg ip. None of the changes in animals treated with PTX differed significantly (Student's t-test; $P>0.05$) from those which occurred in vehicle-treated animals.

Rapid (4 min) iv infusions of PTX isethionate, 2.5, 5, and 10 mg/kg (administered sequentially at 30 min intervals) produced dose-dependent decreases in mean arterial blood pressure (monitored from a femoral artery) and increases (up to 12 ± 8%) in heart rate of dogs anesthetized with allobarbital and urethane. The largest decrease in blood pressure (41 ± 9%) occurred after the 10 mg/kg dose (total cumulative dose = 17.5 mg/kg) and lasted for 50 minutes. Such marked hypotension was not observed when PTX was administered at a slower rate. In an additional study in 3 animals, there were only minor decreases (up to 11%) in mean arterial blood pressure and increases (up to 17%) in heart rate during the 110-minute period following the start of a 10 mg/kg iv infusion of PTX isethionate administered over 30 minutes (approximately).

Respiratory depression as indicated by decreases in minute volume did not occur in anesthetized dogs ($N=3$) after rapid (4 min) iv infusions of PTX isethionate, 2.5, 5, and 10 mg/kg iv (total dose = 17.5 mg/kg iv). Changes in minute volume after the 2.5 and 5 mg/kg doses of PTX were minor and transient. Increases (up to 57%) in minute volume occurred after the 10 mg/kg dose; the magnitude of this change was similar to that seen in control animals infused with vehicle (propylene glycol:water, 1:1, vol/vol). Lesser increases in minute volume occurred after slow (approximately 30 min) iv infusions of PTX, 10 mg/kg.

No arrhythmias were noted in the electrocardiograms of dogs infused with PTX and, after 17.5 mg/kg iv, there were only minor changes (up to 17%) in depressor or pressor responses elicited by iv acetylcholine and norepinephrine. Although pressor responses to bilateral carotid occlusion (30 sec) and depressor responses to electrical stimulation of the right vagus nerve were inhibited (up to 34%) after a total (cumulative) dose of 17.5 mg/kg iv, these effects were only marginally greater than the changes seen in dogs infused with vehicle.

Ancillary Studies

PTX isethionate (100 mg/kg orally) slightly inhibited (25%) the increase in pleural exudate induced in rats ($N=5$) 3 hours after the intrapleural administration of carrageenan (*32*). This acute anti-inflammatory activity of PTX was much weaker than aspirin, which produced 58% inhibition at 30 mg/kg by mouth.

In contrast to reference analgesics, PTX isethionate (100 mg/kg ip) failed to inhibit the hyperalgesia induced in the hind paws of rats ($N=5$) 30 minutes after intraplantar administration of trypsin (*33*).

Significant diuresis did not occur at 2, 5, or 24 hours after the administration of PTX isethionate (20 or 200 mg/kg ip) to saline-loaded mice.

The results from these studies indicate that PTX has only minor pharmacodynamic actions. The studies performed did not reveal either additional therapeutic potential or side effects to the use of this agent at the dosing regimens proposed for man.

ANIMAL TOXICOLOGY

The toxicological evaluation of PTX isethionate was conducted in rats and dogs that received daily oral doses for 1, 5, or 90 days or daily iv doses for 1 or 5 days.

Single-, 5-, and 90-Dose Oral Toxicity Studies in Rats

PTX isethionate was suspended in 40% propylene glycol and administered by gavage to groups of male and female rats at various doses for either 1 or 5 days. The combined male and female doses which were lethal to 10%, 50%, and 90% of animals were 764, 1168, and 1572 mg/kg, respectively, after single oral doses and 80, 131, and 181 mg/kg/day, respectively, after 5 daily doses. Clinical signs of toxicity that preceded death included: weight loss, nasal discharge, soft feces, and urine-stained genital area.

Clinical laboratory data from the 5 dose oral toxicity study revealed that decreased WBC counts, lymphocyte counts, total serum protein, albumin, globulin, and SGPT occurred on the first day after dosing among rats given 60 mg/kg/day for 5 days. These abnormalities as well as decreased absolute neutrophils, SGOT, and alkaline phosphatase were seen on the first day after dosing among rats given 120 mg/kg/day for 5 days and 180 mg/kg/day for 5 days. Reductions of the RBC count, hemoglobin, and hematocrit associated with increases of nucleated RBC, platelets, WBC, neutrophils, lymphocytes, mean cell volume (MCV), and the presence of erythrocytic polychroma were observed on day 15 among female rats receiving 120 mg/kg/day for 5 days.

Histopathologic findings were noted in animals that died, but no or only healing lesions were observed in animals surviving to scheduled killing 29 days after treatment; thus changes were reversible. The cecum was the only tissue examined in the low-dose (60 mg/kg/day for 5 days) animals surviving to killing. Other histopathologic changes were found in the bone marrow (hypoplasia with congestion and hemorrhage), intestine (from maturation arrest to ulcerative enteritis with the cecum affected most severely), spleen and thymus (involution or atrophy), and liver (subacute or chronic pericholangitis in some animals secondary to cecal ulcerations).

Initial doses of PTX for the 90-day oral toxicity study were 0.5, 2.5, and 12.5 mg/kg/day; however, the doses were increased to 5, 15, and 45 mg/kg/day, respectively, on day 33 due to a lack of obvious drug toxicity at that time. Doses were administered by gavage as a solution in 10% propylene glycol. Drug related effects were limited to the intermediate- and high-dose groups and occurred only after the doses were increased. No drug-related effects were noted for the low-dose group (5 mg/kg/day). Clinical signs and laboratory data for the 15- and 45- mg/kg/day groups were analogous to those reported for the 5-dose oral study.

The lowest doses that caused toxic effects in the 1-, 5-, or 90-day oral toxicity studies in rats are summarized in table 5.

Single-, 5-, and 90-Dose Oral Toxicity Studies in Dogs

In the single-dose oral toxicity study in dogs, PTX isethionate in gelatin capsules was given to groups of 2 male and 2 female Beagle dogs at doses of 0, 24, 240, and 480 mg/kg. Clinical observations of those dogs receiving 24 mg/kg revealed reduced food consumption and weight loss in 1 of the 4. These changes were slight and reversible. Dogs receiving 240 mg/kg experienced emesis and changes in fecal consistency; slight and reversible decreases in food consumption were also observed among these animals. Both female dogs receiving 480 mg/kg died or were killed moribund by the seventh day after treatment. Both male dogs were killed on the eighth day. All dogs developed emesis, dehydration, lethargy, and changes in fecal consistency. Hematologic and biochemical abnormalities were

TABLE 5.—Toxic effects of PTX given orally to rats and dogs

	Lowest dose effect noted in					
	Rat			Dog		
Effect	1 dose	5 doses	90 doses	1 dose	5 doses	90 doses
Lowest dose tested, mg/kg/day	500	60	5	24	2.5	0.5
Highest dose tested, mg/kg/day	1100	180	45	480	25	2.5
Death or moribund killing	700	60	45	480	25	2.5
Anorexia or reduced food consumption	500	60	45	24[a]	12.5	2.5
Weight loss or decreased rate of gain	500	60	45	24[a]	2.5	2.5
Diarrhea or soft feces	900	180	45	240[a]	2.5	2.5
Emesis	—	—	—	480	12.5	2.5
Dehydration	700	120	45	480	25	2.5
Decreased WBC	—	60	15	480	2.5	2.5
Decreased segmented neutrophils	—	120	15	480	2.5	2.5
Decreased lymphocytes	900	60	15	—	12.5	2.5
Decreased monocytes	—	—	—	—	25	2.5
Decreased eosinophils	—	—	—	—	12.5	2.5
WBC, rebound	1100	120	—	—	—	—
Decreased RBC and related	—	120	45[a]	—	25	2.5
Hemoconcentration	—	—	45[a]	—	25	2.5
Decreased sodium	—	—	45[a]	—	12.5	2.5
Decreased potassium	—	—	—	—	25	2.5[b]
Decreased alkaline phosphatase	—	120	45	—	—	2.5
Increased alkaline phosphatase	1100[c]	—	—	480	12.5	2.5
Decreased SGOT	—	60	45	—	—	—
Increased SGOT	—	—	—	480	25	—
Decreased SGPT	—	60	45	—	—	—
Decreased total protein	—	60	45	—	—	2.5
Decreased albumin	—	60	45	—	—	—
Decreased globulin	—	60	—	—	—	—
Increased BUN	—	—	45	480	12.5	2.5
Increased creatinine	—	—	45	—	25	2.5
Increased total bilirubin	—	—	—	480	12.5	2.5
Bone marrow hypocellularity	700	120[d]	15[e]	480	12.5	2.5
Epithelial maturation arrest:						
Tongue	—	—	—	480	12.5	—
Esophagus	—	—	—	—	12.5	2.5
Stomach	—	—	—	—	12.5	—
Intestine	700	60	15	480	12.5	2.5
Atrophy-involution thymus, spleen	700	120[d]	45	480	12.5	2.5

[a] Equivocal.

[b] Increased.

[c] Rebound.

[d] 60-mg/kg/day dose group not examined.

[e] Slight maturation arrest.

those associated with antifolate toxicity. Thus, the lethal single-oral dose of PTX in the dog is 480 mg/kg while the lowest oral dose observed to produce drug induced pathologic alterations in clinical parameters is 24 mg/kg.

In the 5-dose oral toxicity study, groups of 2 male and 2 female dogs were given PTX isethionate in gelatin capsules at doses of 2.5, 12.5, and 25 mg/kg/day for 5 consecutive days. Dogs receiving 2.5 mg/kg/day for 5 days developed diarrhea and weight loss (males only), as well as slightly decreased total WBC and absolute neutrophil counts. All these effects were completely reversible. Dogs receiving 12.5 mg/kg/day for 5 days showed weight loss, anorexia, emesis, diarrhea, and decreased activity and antifolate-related abnormalities in clinical and histopathologic parameters. Both male dogs receiving 25 mg/kg/day for 5 days died 3 days after treatment ended. All dogs in this group exhibited lethargy, anorexia, emesis, diarrhea, dehydration, and weight loss. Clinical and histopathologic changes were similar to those observed for the high-dose group of dogs in the 90-day study described next.

Groups of 5 male and 5 female dogs were given 0.1, 0.5, or 2.5 mg/kg/day of PTX isethionate in gelatin capsules for 91 to 92 consecutive days. The drug was administered as a 10% weight to weight blend in starch 1500. Emesis was observed at the low dose, but there were no other significant changes in histopathology and clinical signs or chemistry. At the middle dose, there were no drug-related changes in body weight, food consumption, electrocardiography, or clinical chemistry; however, drug-related decreases, which reversed during the 29-day post-dose period, in RBC counts, hemoglobin, and packed cell volume (PCV) were noted in 1 female on day 86. Other individual susceptibilities to the antifolate effects of PTX were noted in the middle-dose group. Mild myeloid-series maturation arrest was present in 1 of 2 male and 1 of 2 female dogs at the end of the post-dose interval. In addition moderate thymic atrophy was noted in 1 of 2 female dogs post-dose. In contrast only moderate thymic atrophy was noted in 1 of 6 animals killed at the end of dosing. The findings in the post-dose animals were believed to indicate the partial reversal of these changes from the end of dosing in these animals rather than an indication of delayed toxicity.

In the group of dogs that received 2.5 mg/kg/day, 3 male and 4 female dogs died during dosing or were killed in moribund condition between days 14 and 86. The natural or induced deaths were preceded by emesis, diarrhea-like fecal changes, and dehydration. Significant weight losses (15 to 33%) occurred in all dogs with the exception of 1 male dog. Food consumption and body weight returned to normal during the post-dose period. Primary drug-related changes in the hematology were limited to decreases in RBC counts, hemoglobin, PCV and total WBC counts in both males and females. The incidence of the changes was not 100%, indicating variable individual susceptibility to the drug. Primary changes in clinical chemistry were limited to slight decreases in the total protein, albumin, and globulin levels. Additional changes noted only prior to moribund killing were considered secondary physiologic changes and included increased levels of alkaline phosphatase, BUN, creatinine, bilirubin, and potassium, and decreased sodium. Histopathologic effects were consistent with known changes caused by antifolate drugs including epithelial maturation arrest in the esophagus, intestines, and gall bladder; hematopoietic maturation arrest and testicular atrophy in 1 male dog. Additional involutionary changes in the thymus and spleen were considered to be possible primary and/or secondary changes.

The lowest doses that caused toxic effects in the 1-, 5-, or 90-day oral toxicity studies in dogs are summarized in table 5.

Toxicity Studies in Rats and Dogs With PTX iv Formulations

Prior to initiation of the toxicity studies with iv doses of PTX isethionate, blood compatibility studies were conducted. An 11 mg/ml solution of PTX isethionate in propylene glycol:water (50:50) was diluted with 5% aqueous dextrose to final concentrations of 0.2, 0.25, 0.5, 0.75, 1.0, and 10 mg/ml. These solutions were then added to tubes containing either type O human plasma or 50% erythrocyte suspensions in normal saline. The final ratios of drug solution to plasma or RBC suspensions were intended to represent those achievable by assuming instantaneous iv administration of given doses of drug solutions to patients with blood and plasma volumes of 70 and 40 ml/kg, respectively. From the standpoints of protein flocculation, drug precipitation, and erythrocyte hemolysis, a 0.5 mg/ml solution of PTX isethionate (equivalent to 0.36 mg/ml PTX free base) was demonstrated to be comparable to normal saline under conditions much more severe than likely to be encountered under conditions of recommended clinical use. For the single- and 5-dose iv toxicity studies, a 1 mg/ml solution (the highest concentration of PTX in the in vitro studies without significant hemolysis or precipitation) was used to keep volumes in a practical range.

Single- and 5-Dose iv Toxicity Studies in Rats

Because of limitations due to infusion volumes, the highest iv doses that could be given practically to rats were 50 mg/kg single doses and 40 mg/kg/day for 5 days; in both cases these doses were less than the dose lethal to 10% of animals.

Single- and 5-Dose iv Toxicity Studies in Dogs

Preliminary iv studies in dogs with PTX concentrations that were considered compatible with blood indicated it would not be practical to evaluate single-dose toxicity to lethality because of the extremely large infusion volumes that would be required. It was demonstrated, however, that single doses of 5 mg/kg of a 0.2 mg/ml drug solution were most likely not associated with any toxic effects.

In the 5-dose iv toxicity study in dogs, groups of 4 male and 4 female beagle dogs were given 1.25 or 5.00 mg/kg/day of PTX isethionate iv for 5 consecutive days. Drug-related effects in the low-dose group were equivocal to mild, present in a minority of animals, and reversible. One or more clearly drug-related effects analogous to those observed in the dogs that received subchronic oral doses were observed in the dogs that received 5 mg/kg/day. Thus, the 1.25 mg/kg dose of PTX given for 5 days was considered a minimal effect dose while the 5 mg/kg/day dose given iv for 5 consecutive days was associated with moderately severe antifolate toxicity that was largely reversible by 60 days after cessation of treatment.

PREVENTION OF PIRITREXIM TOXICITY

Studies in Cell Culture

The antifolate effect of PTX on sarcoma 180 cells in culture was completely reversed by a combination of thymidine, hypoxanthine, adenosine, and either uridine or cytidine, whereas for MTX only thymidine and hypoxanthine were necessary (*8*). In other reversal studies using sarcoma 180 cells, the toxic effects of MTX were completely prevented by the addition of calcium leucovorin at a concentration 10-fold higher than that of MTX, but the toxic effects of PTX were only partially reversed by calcium leucovorin. When the molar concentration of calcium leucovorin was 250-fold in excess of that of PTX, cell growth in the presence of PTX was only about 60% of the growth in control cultures. The cytotoxic effects of PTX, however, could be prevented by the combination of thymidine and small amounts of calcium leucovorin. In the presence of thymidine, calcium leucovorin or N^5-methyltetrahydrofolate at concentrations ½ that of PTX completely prevented the cytotoxicity of PTX. The same pattern of reversal and partial protection by calcium leucovorin was observed also with L1210 and Walker 256 cells (*8*); however, calcium leucovorin alone was found to reverse the effect of PTX on human WIL-2 cells (*34*).

Studies in Rats and Dogs

In contrast to the in vitro results, rats given a toxic dose of PTX, 100 mg/kg ip, were rescued by administration of calcium leucovorin alone (200 mg/kg, twice daily for 2 days starting 24 hours after the PTX dose), whereas thymidine alone (500 mg/kg, 3 times daily for 3 days starting at the time of PTX dosing) did not prevent lethality (*8*).

Similarly, the lethal toxicity of PTX (25 mg/kg/day orally for 5 consecutive days) in 2 dogs was prevented by calcium leucovorin (0.75 or 3.0 mg/kg) given hourly for 4 doses starting 1 hour after PTX on all dose days. A third dog given the same PTX dose without calcium leucovorin was sacrificed moribund 1 day postdose.

In a second study, 9 dogs were treated with PTX as above. Pairs of dogs were given calcium leucovorin doses of 0.75 or 3.0 mg/kg 4 times daily as above, but were started on calcium leucovorin after PTX doses on either days 2, 3, 4, or 5, respectively. As a control, leucovorin was not given to the ninth dog. Clinical signs, food consumption, and WBC counts were recorded periodically and animals were sacrificed when moribund or 15 days postdose for necropsy and histological examination of the intestinal tract (table 6). With one exception, the dog given the 0.75 mg/kg dose of calcium leucovorin starting 1 hour after the second daily dose of PTX, calcium leucovorin prevented and/or rescued the dogs from the lethal effects of PTX. It is significant that even dogs that did not receive calcium leucovorin until after the fifth dose of PTX were rescued.

SUMMARY

PTX is a potent selective inhibitor of DHFR that enters tissues and tumor cells rapidly by passive diffusion. It is an effective inhibitor of the growth of certain cancer cells that are resistant to MTX because of impaired transport or overproduction of DHFR. Disposition studies have shown that PTX penetrates more extensively into tissues than MTX, but toxicity is reversed readily, probably because PTX is not retained as polyglutamate metabolites. When administered to animals orally or iv (with appropriate drug concentrations and rates), the adverse effects from PTX are limited primarily to antifolate toxicity and reversed by calcium leucovorin. Furthermore, the general pharmacological profile indicated that PTX should be free of CNS, cardiovascular, and respiratory side effects at projected clinical doses. There are important differences in toxicity and pharmacokinetics of PTX and MTX, suggesting that the 2 compounds may have very different profiles of clinical use and anticancer activity.

REFERENCES

(*1*) Grivsky EM, Lee S, Sigel CW, et al: Synthesis and antitumor activity of 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methyl-pyrido[2,3-D]pyrimidine. J Med Chem 23:327–329, 1980.

(*2*) Bertino JR, Sawicki WL, Moroson BA, et al: 2,4-Diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quin-

Table 6.—Prevention and rescue of toxicity in dogs given PTX 25 mg/kg/day for 5 days

			WBC values, × 10^3/mm^3			
Animal	Leucovorin given, days	Leucovorin dose, mg/kg	Pretest	Nadir[a]	Clinical signs[b]	Intestinal maturation arrest
1[c]	–	–	8.2	3.9	+	+
2[c]	–	–	11.9	5.4	+	+
3	1–5	0.75	11.1	7.4	−	−
4	1–5	3.0	10.3	6.3	−	−
5[c]	2–5	0.75	12.8	9.6	+	+
6	2–5	3.0	12.4	11.4	+	−
7	3–5	0.75	12.2	9.8	+	−
8	3–5	3.0	12.4	8.2	+	−
9	4–5	0.75	17.9	15.3	+	−
10	4–5	3.0	16.5	10.2	+	−
11	5	0.75	12.3	9.0	+	−
12	5	3.0	8.7	8.5	+	−

[a] Nadir occurred on either post-treatment day 1 or 3.

[b] + = One or more of the clinical signs (emesis, diarrhea, decreased activity or food consumption) were observed; − = no clinical sign observed.

[c] Killed moribund.

azoline (TMQ), a potent non-classical folate antagonist inhibitor. I. Effect on dihydrofolate reductase and growth of rodent tumors in vitro and in vivo. Biochem Pharmacol 28:1983–1987, 1979.

(*3*) Scanlon K, Ohnuma T, Lo RJ, et al: Effects of 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido(2,3-D)pyrimidine (BW 301U) on methotrexate (MTX)-resistant human acute lymphoblastic leukemia (ALL) cell lines. Proc ASCO 23:12, 1982.

(*4*) Sedwick WD, Hamrell M, Brown OE, et al: Metabolic inhibition by a new antifolate, 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-D]pyrimidine (BW 301U), an effective inhibitor of human lymphoid and dihydrofolate reductase-overproducing cell lines. Mol Pharmacol 22:766–770, 1982.

(*5*) Taylor IW, Slowiaczek P, Friedlander ML, et al: Selective toxicity of a new lipophilic antifolate, BW 301U, for methotrexate-resistant cells with reduced drug uptake. Cancer Res 45:978–982, 1985.

(*6*) Sigel CW, Blum MR, Woolley JL, et al: Preclinical evaluation of BW 301U, 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-D] pyrimidine, a lipophilic inhibitor of dihydrofolate reductase. Proc Am Assoc Cancer Res 25:310, 1984.

(*7*) Weir EC, Cashmore AR, Dreyer RN, et al: Pharmacology and toxicity of a potent "nonclassical" 2,4-diaminoquinazoline folate antagonist, trimetrexate, in normal dogs. Cancer Res 42:1692–1702, 1982.

(*8*) Duch DS, Edelstein MP, Bowers SW, et al: Biochemical and chemotherapeutic studies on 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-D]pyrimidine (BW 301U), a novel lipid-soluble inhibitor of dihydrofolate reductase. Cancer Res 42:3987–3994, 1982.

(*9*) Burchall JJ: Dihydrofolate reductase. *In* Inhibition of Folate Metabolism in Chemotherapy (Hitchings G, ed). Berlin: Springer-Verlag, 1983, pp 55–74.

(*10*) Stickney DR, Simmons WS, DeAngelis RL, et al: Pharmacokinetics of pyrimethamine (PRM) and 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (DDMP) relevant to meningeal leukemia. Proc Am Assoc Cancer Res 14:52, 1973.

(*11*) Cavallito JC, Nichol CA, Brenckman WD, et al: Lipid-soluble inhibitors of dihydrofolate reductase I. Kinetics, tissue distribution, and extent of metabolism of pyrimethamine, metoprine, and etoprine in the rat, dog, and man. Drug Metab Dispos 6:329–337, 1978.

(*12*) Price LA, Hill BT, Goldie JH: Methodichlorophen as an anti-tumor drug. Br Med J 2:20–21, 1975.

(*13*) Miller DS, Rundles RW, Nichol CA, et al: Phase I/II experience with a lipid-soluble folate antagonist: 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (DDMP). Proc Am Assoc Cancer Res, ASCO 17:263, 1976.

(*14*) Price LA, Hill BT, Goldie JH: DDMP and selective folinic acid protection in the treatment of malignant disease: A further report. Clin Oncol 3:281–286, 1977.

(*15*) Hill BT, Price LA: DDMP (2,4-diamino-5-(3′,4′-dichlorophenyl)methylpyrimidine). Cancer Treat Rev 7:95–112, 1980.

(*16*) Alberto P, Brugarolas A, Hansen HH, et al: Phase II study of diamino-dichlorophenyl-methylpyrimidine (DDMP) with folinic acid (CF) protection and rescue. Eur J Cancer 16:1243–1249, 1980.

(*17*) Currie VE, Kempin SJ, Young CW: Phase I trial of metoprine in patients with advanced cancer. Cancer Treat Rep 64:951–956, 1980.

(*18*) Duch DS, Edelstein MP, Nichol CA: Inhibition of histamine metabolizing enzymes and elevation of histamine (HA) levels in tissues by lipid-soluble anticancer folate antagonists. Mol Pharmacol 18:100–104, 1980.

(*19*) Marshall M, Von Hoff D, Chacko A, et al: Effects of drug concentration, exposure time, and serum dialysis on antitumor activity of BW 301U in the human tumor cloning assay. Proc Am Assoc Cancer Res 26:364, 1985.

(*20*) Duch DS, Sigel CW, Bowers SW, et al: Lipid-soluble inhibitors of dihydrofolate reductase: Selection and evaluation of the 2,4-diaminopyrimidine BW 301U and related compounds as antitumor agents. *In* Current Chemotherapy and Infectious Disease (Nelson JD, Grassi C, eds). Washington, DC: American Society of Microbiology 1980, pp 1597–1599.

(*21*) Scheufler E, Zetler G, Iven H: Pharmacokinetics and organ distribution of methotrexate in the rat. Pharmacology 23:75–81, 1981.

(*22*) Foss RG, Sigel CW: Lipid-soluble inhibitors of dihydrofolate reductase III: Quantitative thin-layer and high performance liquid chromatographic methods for measuring plasma concentrations of the antifolate, 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-D]-pyrimidine. J Pharm Sci 71:1176–1178, 1982.

(*23*) Chen ML, Chiou WL: Tissue metabolism and distribution of methotrexate in rabbits. Drug Metab Dispos 10: 706–707, 1982.

(*24*) Duch DS, Lee C-L, Edelstein MP, et al: Biosynthesis of tetrahydrobiopterin in the presence of dihydrofolate reductase inhibitors. Mol Pharmacol 24:103–108, 1983.

(*25*) Duch DS, Bowers SW, Reinhard JF, et al: CNS-toxicity of dihydrofolate reductase (DHFR) inhibitors is not due to effects on tetrahydrobiopterin (BH_4) synthesis. Proc Am Assoc Cancer Res 26:235, 1985.

(*26*) Upton RA: Simple and reliable method for serial sampling of blood from rats. J Pharm Sci 64:112–114, 1975.

(*27*) Sigel CW: BW 301U. Drugs of the Future 10:108–111, 1985.

(*28*) Metzler CM, Elfring GL, McEwen AJ: A package of computer programs for pharmacokinetic modeling. Biometrics 30:562–563, 1974.

(*29*) Chance MRA, Lobstein H: The value of the guinea pig corneal reflex for tests of surface anesthesia. J Pharm Exp Ther 82:203–210, 1944.

(*30*) Dawes GS: Synthetic substitutes for quinidine. Br J Pharmacol 1:90–111, 1946.

(*31*) Blinks JR: Evaluation of the cardiac effects of several β-adrenergic blocking agents. Ann NY Acad Sci 139(3): 673–685, 1967.

(*32*) Vinegar R, Truax JF, Selph JL: Some quantitative temporal characteristics of carrageenan-induced pleurisy in the rat. Proc Soc Exp Biol Med 143:711–714, 1973.

(*33*) Vinegar R, Truax JF, Selph JL: Quantitative comparison of the analgesic and anti-inflammatory activities of aspirin, phenacetin and acetaminophen in rodents. Eur J Pharmacol 37:23–30, 1976.

(*34*) Laszlo J, Iland HJ, Sedwick WD: Overcoming methotrexate resistances by a lipophilic antifolate (BW 301U): From theory to models to practice. Adv Enzyme Regul 24:357–375, 1986.

Initial Clinical Studies of Piritrexim[1]

J. Laszlo,[2,*] W. D. Brenckman, Jr.,[2] E. Morgan,[2] Neil J. Clendeninn,[3] T. Williams,[3]
V. Currie,[4] and C. Young[4,5]

ABSTRACT—Piritrexim (PTX) is a second-generation, lipid-soluble inhibitor of dihydrofolate reductase (DHFR). Metabolic inhibition occurs within seconds after rapid diffusion into human cancer cells. We describe the initial phase I studies with iv and oral forms of this drug given on a daily basis for 5 days to patients with cancer. The dose-limiting toxicity is primarily hematologic (leukopenia, granulocytopenia, thrombocytopenia), but phlebitis is also encountered with iv administration and gastrointestinal problems (nausea, vomiting) with oral administration. Oral toxicity can be reduced by giving the daily dose in 2 divided doses. The maximum tolerated dose (MTD) for the iv route is 170 mg/m^2 per day for 5 days; for the oral route it is 480 mg/m^2 per day for 5 days. Unlike an earlier lipid-soluble folate antagonist, piritrexim did not cause neurologic or histamine-like disorders.—NCI Monogr 5:121-125, 1987.

Classical inhibitors of DHFR have become well established in the treatment of various types of cancer (choriocarcinoma, leukemia, head and neck cancer, etc.). The limitation of drugs such as methotrexate (MTX) is the emergence of drug resistance in the surviving population. Lipid-soluble folate antagonists are a potential solution to at least some forms of MTX resistance, although first-generation compounds (i.e., metoprine) have been too toxic and impractical to use.

If we were to list the properties of an "ideal" lipid-soluble folate antagonist, they would include a high affinity for DHFR, a half-life similar to MTX, and selective cytotoxicity against MTX-resistant cancer cells. Such a new drug should overcome MTX-transport-deficient mutants as well as overproducers of normal DHFR and MTX-resistant DHFR that is collaterally sensitive to the study drug. In addition, an ideal agent would not have non-folate-related organ toxicity, its own toxicity would be preventable and reversible by leucovorin, and it would have good penetration into sanctuary areas (i.e., gonads, CNS).

With respect to these properties, we believe that PTX is an excellent candidate for clinical evaluation, and in a previous paper in this symposium, Dr. Carl Sigel has given some of the background for this conclusion. Our laboratory studies show that PTX is taken up virtually instantly by many types of human cells and is independent of active transport, that it has an affinity for DHFR which is comparable to that of MTX, and that it is capable of inhibiting many types of MTX-resistant cells (*1,2*). Also, we have found that antifolates such as PTX and MTX cause misincorporation of deoxyuridine into hot spots in the DNA, which in turn causes fragmentation of DNA and DNA-protein cross-linking (*3-5*). Furthermore—and this is relevant to later sessions of this symposium on 5-fluorouracil and leucovorin—the cytotoxicity of antifolates such as PTX is augmented by increased concentrations of deoxyuridine in the medium (*6*).

Some points pertaining to documentation of these claims will be illustrated. Figure 1 shows the inhibition of incorporation of labeled deoxyuridine into DNA in (human) WIL-2 cells by 1 μM of MTX (Panel A). This is a well-known time- and temperature-dependent process. By contrast, the center panel illustrates that PTX inhibits deoxyuridine incorporation virtually instantly and does so by a temperature-insensitive reaction that is similar to that of metoprine (Panel C). Table 1 shows that at the same concentration of MTX and PTX (1 μM), there is comparable inhibition of deoxyuridine incorporation into DNA in different types of human leukemic cells as well as immature normal leukocytes (*1*).

After this brief background of our preclinical work, we will review the clinical studies to date which involve a series of phase I and II protocols with intravenous and oral PTX (primarily the joint phase I trials at Duke and Memorial Sloan-Kettering). These studies have been done with 5 daily infusions or oral medication. Other studies have been initiated with single doses and weekly schedules. This presentation will highlight the initial studies of toxicity and pharmacology for the iv and oral formulations. The toxic manifestations are quite repetitive and will mainly be illustrated by the iv trials. Both formulations have some anticancer activity, and the types of cancer in which an objective response has been achieved in these protocols have included malignant melanomas, squamous cell carcinomas (of lung and head and neck), adenocarcinomas of lung and colon, and a malignant fibrous histiocytoma.

PHASE I STUDIES—INTRAVENOUS

The MTD and dose-limiting toxicity (DLT) have been defined with the injectable formulation of PTX given once daily for 5 consecutive days. In patients who received the MTD, approximately 170 mg/m^2/day for 5 days, the major

---

ABBREVIATIONS: DHFR = dihydrofolate reductase; DLT = dose-limiting toxicity; MTD = maximum tolerated dose; MTX = methotrexate; PTX = piritrexim.

---

[1] Supported in part by NIH grant CA-11265 and by the Burroughs Wellcome Co.

[2] Duke Medical Center, Durham, NC.

[3] Burroughs Wellcome Co., Research Triangle Park, NC.

[4] Memorial Sloan-Kettering Cancer Center, New York, NY.

[5] We appreciate the assistance of Mr. Al Guaspari and Ms. Mary Collier in the organization of this project.

* *Reprint requests to:* John Laszlo, M.D., American Cancer Society, 90 Park Avenue, New York, NY 10016.

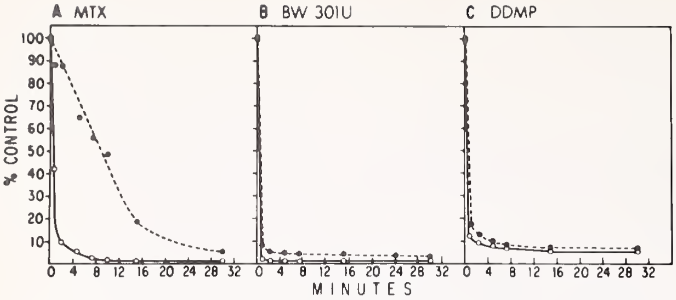


FIGURE 1.—Effect of temperature on onset of inhibition of deoxyuridine incorporation. MTX, BW 301U, and DDMP. WIL-2 cells (2×10^6 cells/ml) were suspended in minimal essential medium (MEM) containing 10% dialyzed fetal calf serum. The cells were then incubated at 37°C (○) or 23°C (●) with 1 *μM* MTX (A), 1 *μM* PTX (B), or 1 *μM* metoprine (C). Control incorporation values in drug-free medium for the experiments with MTX and PTX were carried out on the same day and averaged 10,020 cpm at 37°C and 2,125 cpm at 23°C. Control values for the similar metoprine experiment shown in C averaged 23,558 cpm and 7,131 cpm, respectively. At the indicated time points, 200-μl aliquots from each incubation flask were pulse-labeled for 5 min with 2 μCi of deoxyuridine and processed for counting as described (*3*).

(defined as moderate or greater) toxicities were hematologic, skin rash, oral mucositis, and phlebitis. The average nadir of leukopenia and granulocytopenia was at 8 days after the start of treatment. For platelets, the average nadir was at 11 days. The skin rash was erythematous and pruritic: it began on the fifth day of dosing and resolved within 1 week. Mucositis developed during therapy and resolved within 2 weeks. Thrombophlebitis was erythematous, tender and extended the palpable length of the vein being infused, often resulting in an occluded vein. It appeared to be directly related to the concentration of infused drug, being very common (>50%) at 0.36 mg/ml of 5% dextrose in water and uncommon (<10%) at 0.144 mg/ml. No significant abnormalities of renal, hepatic, pancreatic, endocrine, neurologic, or cardiac function have been noted at the MTD. Lack of neurologic toxicity to date has been particularly encouraging in view of earlier experience with metoprine, and because PTX was selected partially on the basis of its minimal effects in blocking histamine degradation in the brain.

Specific details at each dose level are shown in table 2. Infusions were given daily for 5 consecutive days to 8 cohorts of 3 or more patients each, grouped together into 3 sets for purposes of this representation. Patients were closely monitored for all physical and laboratory changes. PTX isethionate was administered at a concentration of 0.144 mg/ml (expressed as the free base) to the first 6 cohorts and at a concentration of 0.36 mg/ml to the last 2 cohorts. Observations were made of clinical signs and symptomatology during the days of drug administration and include continuous electrocardiographic (Holter) monitoring of the first 9 patients and weekly observations of selected hematologic, coagulation, and biochemical (renal and hepatic function) parameters in all patients. Nausea and emesis were uncommon enough not to be listed in the table, but some patients noticed a slightly metallic taste during drug administration.

The length of infusion at the MTD has ranged from 1 to 2 hours using a drug concentration of 0.36 mg/ml (free base) given at a rate of 3.6 mg/minute. Volumes of infusate have ranged from 670 to 1120 ml among patients, depending on body surface area (1.56 to 2.46 m^2). Among 13 patients given the higher concentration, thrombophlebitis was severe in 1 patient, moderate in 1, and mild in 2. Hematologic toxicity (leukopenia, neutropenia, and thrombocytopenia) was most commonly the DLT. PTX was given to 2 patients whose bone marrow was compromised by metastasis, and they are included in the above analysis. Following administration of 80 mg/m^2/day for 5 consecutive days, thrombocytopenia was observed in 1 patient, with a nadir of 57,000/mm^3 on the 12th day following the start of therapy. In the other patient (who received 170 mg/m^2/day for 5 days), granulocytopenia with a nadir of 1232/mm^3 was noted on the 8th day.

Pharmacokinetic determinations were made on serial plasma samples taken on day 5 of the 5-day treatment (Blum R, manuscript in preparation). The mean half-life was between 3.1 and 5.3 hours for each cohort. One patient had moderate liver dysfunction and had a prolonged drug half-life of 13.9 hours; the presence of preexisting liver disease should be a warning. PTX was bound 87.0 to 95.9% to plasma proteins. Concentration achieved in plasma was related to dosage. Normal bone marrow cells are sensitive to metabolic inhibition by PTX both before and immediately after the 5-day course. By contrast, a relative resistance develops to metabolic inhibition by MTX, the subject of a recent report (*7*).

TABLE 1.—Metabolic response of human leukemic, immature-normal, and bone marrow cells to MTX or PTX

Diagnosis	Drug concentration, *μM*	% inhibition of deoxyuridine incorporation into DNA[a] MTX	PTX
1. Acute myelogenous leukemia (AML)	1	56	33
AML	1	86	85
AML	1	56	46
AML	1	54	64
AML	1	55	79
2. Acute myelomonocytic leukemia (AMML)	1	94	97
3. Chronic myelogenous leukemia (CML)	1	96	95
CML	1	84	91
4. Chronic lymphocytic leukemia (CLL)	1	88	84
5. Hairy cell leukemia	1	69	72
6. Myeloproliferative disorder (MPD) or chronic granulocytic leukemia (CGL)	0.5	85	97
7. MPD	1	49	68
8. Leukemoid reaction	1	95	72
9. Normal bone marrow[b]	1	90	72
Normal bone marrow[b]	1	95	91
Normal bone marrow[b]	1	86	90

[a] Cells were isolated, incubated with labeled deoxyuridine for 30 min and the labeled fraction in DNA was determined as previously described (*2*).

[b] Pulse labeled for 90–120 min.

TABLE 2.—Toxicity incidence of PTX injection daily for 5 days (Duke and MSKCC)

Dose range	Toxicity grade	Skin	Stomatitis	Phlebitis	WBC	Granulocytes	Platelet	Hepatic	Renal
11-56 mg/m²/day ($N=12$)									
	1				1				2
	2							1	
	3								
	4								
80-103 mg/m²/day ($N=9$)									
	1	1			2		4	1	2
	2				2		2	1	
	3				2	1			
	4								
144-170 mg/m²/day ($N=13$)									
	1	5	1	2	4	2	2		1
	2			1	2	1	3		
	3			1	3		1		
	4					1			

A phase I study of PTX infusions given once weekly for 4 consecutive weeks is ongoing at San Antonio. Cohorts of 3 or more patients each were given escalating doses up to 400 mg/m². Data reported from this series included 19 treated patients of which 15 had received at least 4 doses (*8*). Tumor types included non-small cell lung—8, head and neck—3, breast—2, unknown primary—3, and other—3. There were 6 deaths on study; 3 were attributed to tumor progression and 3 to sepsis apparently unrelated to drug toxicity. Patients' toxicities included: phlebitis at the injection site—5; altered taste—3; nonhemolytic anemia—3; thrombocytopenia—3; and mild fatigue—3. Infrequent side effects included facial flushing, transient dyspnea, generalized pruritis, elevated serum transaminases, diarrhea, and elevated temperature following infusion. Although no antitumor responses have been seen up to a dose of 311 mg/m², 4 patients had stable disease and received a second course.

PHASE II STUDIES

A phase II study of PTX injection for metastatic colorectal cancer patients, with the MTD (170 mg/m² for 5 days) given every 3 weeks, is currently being completed at Duke University Medical Center. Doses given after week 1 were adjusted for hematologic toxicity. Twenty-three patients have been treated and the study is not fully analyzed, although some antitumor effects have been noted (Laszlo et al., unpublished data).

PHASE II DOSING

Based on the aforementioned studies, our recommended dosing for future phase II trials is 150 mg/m²/day for 5 days in 1500 ml of 5% dextrose in water given over 6 hours at a concentration not to exceed 0.25 mg/ml.

PHASE I ORAL

PTX capsules have been given once daily for 5 consecutive days. At the MTD of 480 mg/m²/day for 5 consecutive days, the major toxicities were similar to the iv route except that there was no phlebitis. There was, however, significant nausea and vomiting. The toxicities encountered were hematologic (moderate or greater): skin rash, oral mucositis, and significant nausea. No significant abnormalities of renal, hepatic, pancreatic, endocrine, CNS, or cardiac functions have been noted at the MTD. Specific details of these studies have been presented (*9-11*) or are awaiting publication. Salient clinical toxicity is given below.

The DLT at the highest dose were gastrointestinal and hematopoietic. Virtually all patients complained of nausea and vomiting, while 3 had oral stomatitis. Four of 9 had significant hematologic toxicity. Leukopenia was life-threatening (<1,000/mm³) in 1, moderate (2,000 to 2,999/mm³) in 2, and mild (3,000 to 3,999/mm³) in 2 others. Granulocytopenia was severe (250 to 499/mm³) in 2 and mild (1,000 to 1,499/mm³) in 1. Thrombocytopenia was severe (25,000 to 49,999/mm³) in 1, moderate (50,000 to 99,999/mm³) in 1, and mild (100,000 to 149,999/mm³) in 2. No abnormalities of the serum electrolytes, amylase, or coagulation tests were observed. The dynamics of granulocyte and platelet fall and recovery were identical to the iv study.

Because of the gastrointestinal toxicity, a separate study was done giving PTX twice daily for 5 days in an effort to decrease nausea, which had occurred in all patients on the once-a-day schedule at MTD. We found comparable toxicity to the daily schedule except that far less gastrointestinal toxicity was experienced.

Pharmacokinetic studies measured over a 12-hour period with the last dose were performed in all patients on the single-dose schedule. The principal pharmacokinetic parameters indicate that the serum disappearance half-time was 3 to 5 hours and the time to maximum serum concentration was 1.5 to 2.0 hours. Further details will be reported separately (Blum R, manuscript in preparation).

A separate phase I study is ongoing in Arizona to determine the MTD and DLT when PTX capsules are given once weekly for 4 consecutive weeks. Among the 15 patients given up to 660 mg/m², there has been minimal toxicity. Schedule manipulations are underway to increase the bioavailability of the oral dose.

PHASE II STUDIES

A phase II trial of PTX capsules in non-small cell lung cancer patients is ongoing at the Memorial Sloan-Kettering

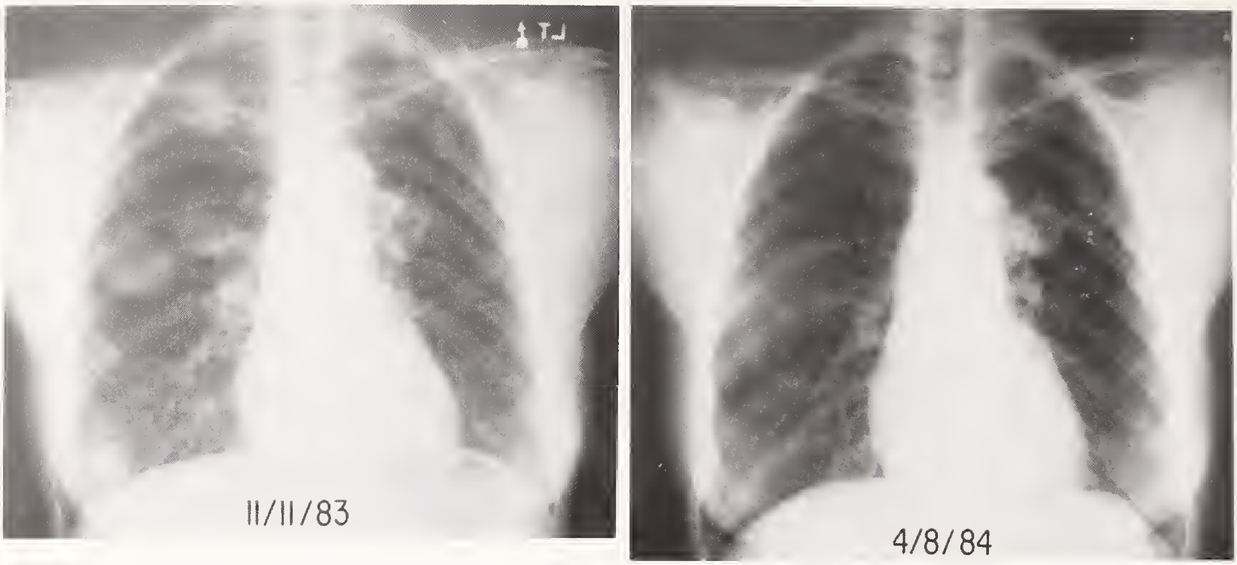


FIGURE 2.—This woman with widely metastatic malignant melanoma participated in the phase I trial of PTX. In 5 mo, the pulmonary and other soft tissue lesions had decreased by more than 50%; this partial remission was maintained for 1 yr in the absence of further treatment.

Cancer Center. Previously untreated patients are receiving 240 mg/m^2 twice daily for 5 consecutive days. Heavily pretreated patients receive no more than 160 mg/m^2 twice daily for 5 days. Analysis of the data is in progress.

Additional phase II trials with the same schedule have begun in metastatic colon cancer and malignant melanoma. Hematologic and gastrointestinal toxicity often necessitate dosage reduction. Attempts to repeat courses every 2 weeks indicate that this may be too soon for complete hematologic recovery in some patients.

PHASE II DOSING

Our recommended oral dose for future phase II trials is approximately 200 mg/m^2 every 12 hours for 5 days and repeated every 3 weeks. Toxicity can be expected in some patients, particularly those who have had extensive prior treatment.

In conclusion, PTX is a novel lipid-soluble antifol that has been tested by over 200 patients in phase I and II trials. We have determined the MTD to be 150 mg/m^2/day for 5 days for iv administration and 200 mg/m^2 twice daily for 5 days for oral administration. The DLT are primarily hematologic, skin rash, oral stomatitis, and phlebitis (iv only). Responses in 5 diseases have been observed. That this drug is of clinical interest is illustrated by a dramatic response in a patient with advanced malignant melanoma who experienced a greater than 50% reduction of lung lesions (fig. 2).

Future directions of research that we think are of interest include determining the importance of schedule and dose on effectiveness, determining whether leucovorin protects and/or rescues from PTX toxicity, determining how PTX will modulate other antimetabolites (including MTX), and defining the tumor types in which PTX is active. It is of considerable theoretical interest to determine the spectrum of cytotoxicity, not only because of the unique physical properties of the drug but also because, unlike MTX, it is not susceptible to being polyglutamated to a storage form.

REFERENCES

(*1*) SEDWICK WD, BIRDWELL R, LASZLO J: Lipid-soluble pyridopyrimidine, BW 301U, with metabolic inhibitory capacity equal to methotrexate in human lymphoblastoid, normal myeloid, human leukemia, and dihydrofolate reductase-overproducing mouse cells. *In* Current Chemotherapy and Infectious Diseases (Nelson JD, Grassi C, eds). Washington, DC: American Society for Microbiology, 1980, pp 1593–1959.

(*2*) SEDWICK WD, HAMRELL M, BROWN OE, et al: Metabolic inhibition by a new antifolate, 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methyl-pyrido[2.3d]pyrimidine (BW 301U) an effective inhibitor of human lymphoid and dihydrofolate reductase-overproducing mouse cell lines. Mol Pharmacol 22:766–770, 1982.

(*3*) SEDWICK WD, KUTLER M, BROWN OE: Antifolate-induced misincorporation of deoxyuridine monophosphate into DNA: Inhibition of high molecular weight DNA synthesis in human lymphoblastoid cells. Proc Natl Acad Sci USA 78:917–921, 1981.

(*4*) RICHARDS RG, BROWN OE, SEDWICK WD: Misincorporation of deoxyuridine in human cells: Consequences of antifolate exposure. *In* The Genetic Consequences of Nucleotide Pool Imbalance (de Serres F, ed). New York: Pergamon Press, 1985, pp 149–162.

(*5*) RICHARDS RG, SEDWICK WD: Consequences of antifolate exposure on DNA integrity and chromatin structure in cultured human cells. Proc Am Assoc Clin Res 26:234, 1985.

(*6*) LASZLO J, ILAND HJ, SEDWICK WD: Overcoming methotrexate resistance by a lipophilic antifolate (BW 301U): From theory to models to practice. *In* Advances in Enzyme Regulation (Weber G, ed), vol 24. New York: Pergamon Press. In press.

(*7*) ILAND HJ, LASZLO J, SEDWICK WD: Paradoxical effect of BW 301U, a lipophilic antifolate, on methotrexate-inhibitable deoxyuridine incorporation by human hematopoietic cells. Cancer Res 45:3962–3968, 1985.

(*8*) SAROSY G, WEISS G, WILLIAMS T, et al: Phase I trial of BW 301U administered on a weekly×4 schedule. Proc ASCO 4:29, 1985.

(*9*) Iland H, Laszlo J, Brenckman W, et al: Preliminary phase I clinical trials and pharmacokinetics of BW301U, a new lipid-soluble folate antagonist. Proc ASCO 3:29, 1984.

(*10*) Laszlo J, Brenckman W, Currie V, et al: Phase I study of BW 301U infusions administered on five consecutive days. Proc Am Assoc Clin Res 26:178, 1985.

(*11*) Brenckman W, Laszlo J, Morgan E, et al: Phase I evaluation of BW 301U administered orally on five consecutive days. Proc ASCO 4:45, 1985.

10-Ethyl-10-Deaza-aminopterin: Structural Design and Biochemical, Pharmacologic, and Antitumor Properties[1]

F. M. Sirotnak,[2,*] F. A. Schmid,[2] L. L. Samuels,[2] and J. I. DeGraw[3]

ABSTRACT—The new folate analog 10-ethyl-10-deaza-aminopterin (10EdAM) was equivalent to methotrexate (MTX) as an inhibitor of dihydrofolate reductase, but was more effectively transported and polyglutamylated in most tumor cells. Also, the transport and polyglutamylation of 10EdAM in tumor cells vis-a-vis normal proliferative tissue is substantially increased compared to MTX, favoring much greater accumulation of 10EdAM as cytotoxic polyglutamates in some of these tumor cells. 10EdAM was superior to MTX against 4 of 6 murine ascites tumors (L1210, S180, Ehrlich and Tapper) and far superior against 4 of 6 solid murine tumors (S180, Tapper, E0771 mammary AC, T241 fibrosarcoma). 10EdAM produced 10% to 30% complete regressions against S180, E0771 and T241 tumors. Both agents showed similar activity against P288 and 1498c leukemias and the Lewis lung tumor, but were inactive against B16 melanoma. Marked superiority of 10EdAM compared to MTX was also shown against the following human tumor xenografts: MX-1 (mammary carcinoma), LX-1 (small cell lung carcinoma) and CX-1 (colon carcinoma). 10EdAM produced 30% to 40% complete regressions against the MX-1 tumor.—NCI Monogr 5:127-131, 1987.

For the purpose of designing new 4-amino folate analogs with enhanced selectivity of antitumor action, we have sought to exploit differences between tumor and normal proliferative tissues, particularly in drug-limiting organs, at the level of mediated membrane transport and polyglutamylation of these agents (*1,2*). We have found (*1–3*) that the difference in membrane transport of folate analogs between responsive tumors and normal proliferative tissues in the mouse, which appears to favor greater accumulation of these agents in the former through increased mediated membrane transport, can be accounted for by a difference in specificity for substituents in at least 1 of 3 different sites on the folate molecule (the 5-position, the 10-position or the γ-carboxyl group). Variable specificity for substituents in both the 5- and 10-positions among different tumors was also seen for polyglutamylation (*4,5*). Since substituents in these positions have little effect on the inhibition of the intracellular target, dihydrofolate reductase (*1–3*), new analogs which are modified at these positions could potentially have improved therapeutic activity. We now describe the results of studies characterizing the biochemical, pharmacologic and growth-inhibitory properties of a new member of the 10-deaza-aminopterin (10dAM) series of analogs, 10EdAM. We also present the results of antitumor studies in murine models and in human tumor xenografts which have led to the clinical trial of this agent in cancer patients.

MEMBRANE TRANSPORT AND POLYGLUTAMATION OF 10-ETHYL-10-DEAZA-AMINOPTERIN AND RELATED ANALOGS

On the basis of the above considerations, a new series of folate analogs, the 10-deaza-aminopterins (fig. 1), was synthesized (*6*) and evaluated (*7–10*). The parent compound, 10dAM, showed increased therapeutic activity compared to MTX in some murine ascites tumors, notably sarcoma 180 (*7*). However, one alkyl derivative, 10EdAM, showed vastly increased antitumor selectivity in both ascitic and solid murine tumors and in human tumor xenografts (*9,10*). It appears that some measure of this increased selectivity of this agent compared to the other 2 agents was a result of a further increased differential in membrane transport (tumor versus normal proliferative tissue) and polyglutamylation over MTX and the parent analog, favoring its greater accumulation as a cytotoxic polyglutamate in tumor (*4,5*).

A summary of the biochemical and cytotoxicity studies (*4,5,7,9*) carried out with these 3 agents and aminopterin (AM) is presented in tables 1 to 6. As inhibitors of L1210 cell dihydrofolate reductase at neutral pH (pH=7.3), the 2 new analogs were equivalent (table 1) to MTX and AM. pH-Dependence for inhibition of this enzyme was also found to be similar for all 4 analogs. Influx of these analogs in L1210 cells (table 1) was saturable and efflux was nonsaturable. Values for both influx capacity (V_{max}) and the efflux rate constant were essentially identical for all 4 analogs. In contrast to the nitrogen 10 analogs, these 4 showed no effect of alkylation at carbon 10 on influx. Values for influx K_m of AM, 10dAM, and 10EdAM were 3 to 4 times lower than that of MTX. Consistent with these results, the data show intracellular exchangeable levels for AM, 10dAM, and 10EdAM to be 3- to 4-fold higher than for MTX at an external concentration (1.0 μM) in the limiting range ($<K_m$).

In competition experiments (table 2) measuring the effects of each analog on [^{3}H]MTX transport, values for the

---

ABBREVIATIONS: AM=aminopterin; 10dAM=10-deaza-aminopterin; 10EdAM=10-ethyl-10-deaza-aminopterin; IC_{50}=concentration which causes 50% inhibition of growth; K_i=inhibitory constant; K_m=concentration for half the saturation of influx; MTX=methotrexate; V_{max}=influx capacity.

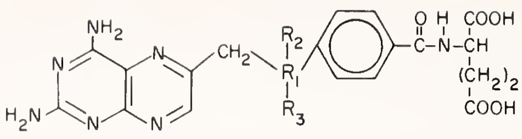
---

[1] Supported in part by grants CA-08748, CA-18856, CA-22764, and CA-28783 from the National Cancer Institute and funds from the Elsa U. Pardee Foundation.

[2] Memorial Sloan-Kettering Cancer Center, New York, NY.

[3] SRI International, Menlo Park, CA.

* *Reprint requests to:* F. M. Sirotnak, Ph.D., Laboratory for Molecular Therapeutics, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.



R_1	R_2	R_3	Analog
N	CH_3	—	methotrexate
C	H	H	10-deaza-aminopterin
C	C_2H_5	H	10-ethyl, 10-deaza-aminopterin

FIGURE 1.—Structural formulas for the 10-deaza-aminopterins and related folate analogs.

influx inhibitory constant (K_i) were derived that were essentially the same as values for the saturability of influx (K_m). Similar data was obtained for S180 cells and Manca cells. Values for K_i for AM and the 10-deaza-aminopterins were essentially the same for each cell type. However, the corresponding values for MTX were not. These were 3- to 4-fold (L1210), 12-fold (S180), and 7-fold (Manca) greater than for the 10-deaza-aminopterins. These data imply greater net accumulation of the new analogs compared to MTX in all 3 cell types.

In other experiments, the initial rate of entry and polyglutamylation of radioactive derivatives of these analogs in these tumor cells was determined (table 3) during growth in culture (*5*). Measurements of polyglutamate formation that are a valid representation of initial rates of formation and accumulation of these metabolites in each cell type were obtained by using concentrations of each analog in the external medium, resulting in comparable rate of entry and level of accumulation. These concentrations were derived from the kinetic data given in tables 1 and 2. Initial rates of intracellular accumulation were similar for all 4 agents, varying only from 1.8 to 2.2 nmol/minute/g dry weight. Intracellular drug levels exceeded the binding equivalence of dihydrofolate reductase within 5 to 10 minutes, at which time polyglutamates of each analog began to appear. Rates of accumulation of these anabolites were constant by 20 minutes and remained so for the next 40 minutes during which measurements were made. Rates of accumulation of polyglutamates were 7- to 18-fold lower than rates of drug entry into these cells. In L1210 cells, these rates varied among analogs in the order AM>10EdAM>MTX>10dAM. From the data, it can be seen that the same relative order and maximum level of substrate activity for these analogs was seen in S180 cells, but the overall differential in relative rates was substantially greater. However, the ranking among analogs was different for Manca cells, 10EdAM≅AM>10dAM>MTX, and the overall level of polyglutamate formation was considerably greater than for L1210 and S180 cells. This data would appear to reflect the extent of differences in substrate preferences for these various analogs by folylpolyglutamyl synthetase as well as inherent capacity for polyglutamate formation in each tissue. However, additional work at the level of the isolated enzyme will be required to establish this point conclusively.

Some validation of the predictiveness of data in tables 1 to 3 for relative antitumor potency was obtained in studies measuring the cytotoxicity of these analogs with a clonogenicity assay (*5*). These data are given in table 4. In these experiments, MTX was the least effective analog. Activity of this analog against L1210 and Manca cells was 8- to 10-fold greater than against S180 cells. However, the overall potency of AM was similar against all 3 cell types, and compared to MTX, at least 12- to 15-fold greater for L1210 and Manca cells and as much as 80-fold greater for S180 cells. Overall, potency for 10EdAM was similar to AM, but individual concentrations which caused 50% inhibition of growth (IC_{50}) were about 2-fold greater. Also, the differences between 10EdAM and MTX were not quite as large. In contrast to results with AM, potencies for 10dAM varied substantially among these cell lines. It was 2- to 4-fold less potent than was AM against Manca and L1210 cells, but 12-fold less potent against S180 cells. These results are seemingly explained by differences in both membrane transport and polyglutamylation, thus reaffirming the probable role of both properties as cytotoxic determinants (*1–14*). In the case of MTX and AM, the higher potencies of the latter in the 3 tumor cell types reflect both higher mediated influx (tables 1 and 2) and polyglutamylation (table 3) of each. Likewise, the variable potency of each of the 4 analogs among the 3 cell types is determined by differences in both biochemical parameters.

In contrast to the results of transport studies carried out with these murine tumors, data from similar studies with the purified proliferative fraction of epithelial cells isolated from mouse small intestine showed substantial differences between the new analogs (*5*). The results are summarized in table 5. Mediated transport of folate analogs in these cells shows kinetics similar to those found in tumor cells (*15*). However, the overall K_m is lower and the V_{max} is substan-

TABLE 1.—Biochemical properties of various folate analogs in L1210 cells

Analog	DHFR inhibition,[a] K_i p*M*	Influx, K_m μM	Influx, V^{37}_{max} nmol/min/g dry weight	Efflux, K^{37} min^{-1}	Net $[drug]_{int}$,[b] μM
AM	2.3±0.2	1.18±0.2	9.83±1.1	0.21±0.02	6.4±0.7
MTX	3.8±0.8	3.84±0.2	7.77±0.9	0.19±0.02	1.8±0.2
10dAM	2.1±0.1	0.93±0.1	8.13±0.1	0.23±0.03	6.8±0.6
10EdAM	2.4±0.2	0.89±0.1	7.80±1.1	0.18±0.02	6.5±0.7

[a] At pH 7.3; DHFR=dihydrofolate reductase.

[b] Intracellular drug concentration less drug bound to dihydrofolate reductase (DHFR=2.92±0.3 nmol/g dry weight) as determined in (*3*). External drug concentration was 1.0 μM.

TABLE 2.—Summary of data on influx properties of folate analogs in various tumor cells

	Mediated-influx K_I[a,b]		
Analog	L1210, μM	S180, μM	Manca, μM
AM	1.08±0.1	1.46±0.2	1.38±0.3
MTX	3.57±0.4	11.8±1.5	6.23±0.5
10dAM	0.93±0.1	0.97±0.2	0.92±0.1
10EdAM	0.89±0.1	0.93±0.1	0.94±0.1

[a] Mean±SE for 3 to 5 individual determinations of competitive inhibition of [^{3}H]MTX influx (*3*).

[b] Values for influx V_{max} (nmol/min/g dry weight) for [^{3}H]MTX were 7.48±0.9 (L1210), 8.23±1.1 (S180), and 2.54±0.2 (Manca). Values for efflux rate constants (min^{-1}) were 0.213±0.03 (L1210), 0.208±0.02 (S180), and 0.211±0.03 (Manca).

TABLE 4.—Cytotoxic effects of various folate analogs against murine and human tumor cells

	Half-maximal concentration[a]		
Analog	L1210 nM[b]	S180, nM	Manca, nM
AM	12.8± 3	15.7± 4	8.9± 2
MTX	148 ±32	1267 ±27	126 ±31
10dAM	43.0± 8	167 ±38	17.0± 3
10EdAM	22.3± 6	30.1± 6	13.7± 3

[a] IC$_{50}$ for reduction in colony formation. Mean of 4 determinations ±SE of the mean.

[b] Clonogenic assay employing 3-hr exposure to drug and dilution of cell suspension in soft agar.

tially higher as is efflux. The results in table 5 show that alkylation at the carbon 10 as well as nitrogen 10 markedly increased K_m. Since no other difference was seen with respect to other kinetic parameters, differences in K_m alone appear to determine relative intracellular accumulation among these analogs (table 5). These data appear to be somewhat predictive of the relative toxicities of these agents in mice, since drug-limiting toxicity [reviewed in (*2,3*)] occurs in this proliferative cellular fraction in small intestine. However, AM is considerably more toxic than the data on transport alone would appear to predict. The extent to which this can be accounted for by a differential in polyglutamylation of these analogs in this tissue is shown in table 6. In these studies, we examined both small intestine and tumor cells for polyglutamates 4 hours after administration of folate analogs to tumor-bearing mice (*4*). MTX and 10dAM were given at 12 mg/kg sc (the approximate tolerated dose) every 2 days for 10 days. Because of differences in tolerance, AM, the most toxic, was given at 3 mg/kg and 10EdAM, the least toxic, at 18 mg/kg. In the small intestine, the accumulation of MTX, 10dAM, and 10EdAM as polyglutamates was approximately the same. The accumulation of AM as a polyglutamate, however, was substantially greater. It should be pointed out that the absolute level of polyglutamation of these analogs in the proliferative fraction of epithelium of small intestine may actually be higher than these results show. Although these analogs may be accumulated in the total epithelial cell population, their polyglutamylation could be restricted to the proliferative compartments.

The results obtained with tumor tissue following administration of drug in vivo were strikingly different (table 6). Polyglutamylation of these analogs was greater overall than in small intestine. Polyglutamylation of AM and 10EdAM was appreciably greater than for the 2 other analogs in S180 cells, but less so in L1210 cells. With the exception of S180 cells, metabolic conversions of MTX and 10EdAM were similar and higher than of 10dAM, which, overall, was polyglutamylated to the least extent. The polyglutamates that accumulated in tumor cells were primarily in the form of di-, tri- and heptaglutamates for all 4 analogs. In small intestine, the accumulated polyglutamates consisted primarily of triglutamates. Taken together with the transport data for epithelial cells (table 5), the lower extent of polyglutamylation of MTX, 10dAM, and 10EdAM in small intestine compared to AM would appear to explain the much greater toxicity of AM in mice. From a comparison of the data on transport (tables 1, 2, and 5) and polyglutamylation (tables 3 and 6) in vitro and in vivo, 10EdAM would appear to be potentially the most efficacious analog in these tumor models. Its transport inward and accumulation as a polyglutamate was the lowest in the proliferative compartment of the drug-limiting organ, which is consistent with its having the least toxic potency of the group. Also, this analog consistently exhibited the greatest differential in transport inward and in subsequent polyglutamylation in tumors versus proliferative epithelium of mouse small intestine.

TABLE 3.—Net intracellular rate of polyglutamate formation

	L1210	S180	Manca
Analog		nmol/min/g dry weight[a]	
AM	0.315±0.4	0.315±0.04	1.09 ±0.1
MTX	0.200±0.02	0.040±0.005	0.423±0.03
10dAM	0.100±0.02	0.013±0.002	0.505±0.05
10EdAM	0.237±0.05	0.079±0.007	1.017±0.06

[a] Mean±SE. Extracellular concentrations employed during growth of these tumor cells were 2 μM [^{3}H]AM, [^{3}H]10dAM and [^{3}H]10EdAM and 10 μM for [^{3}H]MTX. Initial rates of accumulation varied from 1.8 to 2.2 nmol/min/g dry weight for these compounds.

TABLE 5.—Mediated membrane transport of folate analogs in the proliferative fraction of intestinal epithelial cells isolated from mouse small intestine

	Mediated transport[a]			
Analog	Influx, K_m μM	Influx, V_{max}^{37} nmol/min/g dry weight	Efflux, K^{37} min^{-1}	Net [drug]$_{int}$,[b] μM
AM	15.8± 2	55.6±8	0.61±0.08	1.61±0.4
MTX	102 ±19	51.7±9	0.57±0.09	0.25±0.1
10dAM	48.2± 8	59.3±7	0.65±0.06	0.54±0.1
10EdAM	149 ±27	56.2±9	0.62±0.03	0.16±0.04

[a] Mean±SE of 4 separate determinations. Proliferative cells were purified to the extent of 85–90% by nonenzymatic fractionation (*15*) of everted small intestine.

[b] Intracellular drug concentration less drug bound to dihydrofolate reductase (1.23±0.2 nmol/g dry weight) as determined in (*3*). External drug concentration was 1.0 μM.

TABLE 6.—Recovery of folate analogs as polyglutamates in murine tissues 4 hours after in vivo administration sc

Analog	Small intestine	L1210	% as polyglutamate S180	E0771	T241
AM	28.0±5	79.0±9	35.3±5	—[a]	—[a]
MTX	5.0±2	51.0±6	19.1±4	53.5±6	56.3±7
10dAM	3.2±0.6	39.6±6	9.5±2	12.7±3	14.8±4
10EdAM	8.7±1.3	65.0±8	42.8±6	60.6±7	69.2±8

[a] Not determined.

PHARMACOKINETICS OF 10EdAM AND RELATED ANALOGS IN MICE

10EdAM and its parent derivative, 10dAM, exhibited plasma clearance kinetics in mice identical to that of MTX (*4,8*). The approximate half-lives for the excretory and reabsorption phases were, respectively, 26 minutes and 8 hours. A positive correlation between the extent of persistence of exchangeable levels of folate analogs in various murine proliferative tissues (normal and tumorous) and their cytotoxicity for these tissues has been documented in numerous reports from our laboratory [for review see (*2,3*)]. Greater persistence of MTX at pharmacologically effective levels in cells of responsive tumors than in normal proliferative tissues appeared to account for the selectivity of effects against tumor (*2,3*). In more recent studies in mice, evidence was obtained for a larger differential in persistence of 10dAM and, particularly, 10EdAM in tumor versus small intestine compared to MTX for all of the tumor models examined (*4*). These results are consistent with the biochemical data described above and provided a further basis for predicting that 10EdAM would have substantially improved therapeutic selectivity compared to MTX. The details of a more elaborate pharmacokinetic study of 10EdAM in mice, rats, and dogs are provided elsewhere in this volume.

ANTITUMOR PROPERTIES OF 10EdAM AND RELATED ANALOGS

In studies recently published, 10EdAM was found to be markedly superior to the parent derivative, 10dAM, and MTX in a variety of ascitic and solid murine tumor models (*9*). This superiority was demonstrated employing sc dosing alone starting 1 or 2 days after tumor implantation on a variety of schedules, including daily administration for 5 days, every other day for 10 days, and every 3 or 4 days for 3 injections. The relative tolerances in $BD2F_1$ mice on these schedules was basically the same and in the order 10EdAM > MTX > 10dAM. In each case, dose-limiting toxicity occurred in the crypt-cell epithelium of small intestine. On a schedule of every 2 days for 10 days, 10EdAM achieved $\log_{10}$ reduction in tumor burden several-fold greater than MTX against L1210 and S180 ascites tumors. There were also long-term survivors with 10EdAM in both models. 10dAM gave a result intermediate between those obtained with 10EdAM and MTX. 10EdAM was also 2-fold more active than MTX against the Tapper-liver ascites tumor, but both agents gave approximately the same activity against the 1498c myeloid ascites tumor. Using a schedule of once per day for 5 days against the solid forms of the Tapper tumor, some partial regressions were obtained with MTX and 10dAM, but a far greater number extending over a longer period of time were obtained with 10EdAM. On the same schedule, against the E0771 mammary adenocarcinoma, 10dAM was 2-fold and 10EdAM was >10-fold more effective than MTX in retarding tumor growth. Evidence was also obtained for complete regressions (30%), marked effects against metastatic disease and significant increase in survival in the case of 10EdAM, but not 10dAM or MTX. Similar results were obtained with the T241 fibrosarcoma. For the Lewis lung carcinoma, the relative potency of the MTX and 10dAM was about the same and 10EdAM was more active, but overall antitumor effects were more modest. None of the analogs showed activity against the B16 melanoma.

Other studies were recently carried out using a group of human tumor xenografts (*10*). In Swiss nude mice, the approximate dosages lethal to 10% of animals on a schedule of once per day for 5 days were 1.5, 3, and 4.5 mg/kg given sc for 10dAM, MTX, and 10EdAM, respectively. Employing this schedule in one course against the MX-1 mammary adenocarcinoma xenograft starting 2 days after tumor implantation, MTX was virtually inactive (table 7 and fig. 2) and 10dAM was only minimally active, showing slight retardation of growth at tolerated doses. However, 10EdAM showed substantial antitumor activity. At a dose of 2 to 4.5 mg/kg, reduction in tumor volume was seen (table 7 and fig. 2) 1 day after therapy, reaching a nadir 4 days later of 57% to 76% reduction. Also, at these doses of 10dAM a number of animals showed no palpable tumors. Tumor-free mice were observed at 5 days (4 of 9 mice), 12 days (3 of 9) and 26 days (1 of 9) after therapy with 4.5 mg/kg.

Against the LX-1 small-cell lung carcinoma, overall antitumor activity was similar (table 7) to that obtained against the MX-1 tumor. However, treatment with tolerated doses of MTX and 10dAM resulted in some retardation of tumor growth. Again, treatment with 10EdAM (2 to 4.5 mg/kg) resulted in some reduction in tumor volume. At the nadir, 5 days following therapy, the reduction was 20% to 50% of the original tumor. However, unlike the results obtained with the MX-1 tumor, no animals were found to be tumor-free after therapy with this analog. Treatment of the CX-1 tumor with tolerated doses of MTX or 10dAM was essen-

TABLE 7.—Antitumor activity of folate analogs against murine tumors and human tumor xenografts

Tumor	Species	Type	Average reduction in tumor burden[a] MTX	10dAM	10EdAM
L1210	Murine	Lymphoid leukemia	+	++	+++[b]
S180	Murine	Ascites sarcoma	±	++	++++[c]
E0771	Murine	Breast adenocarcinoma	−	−	++++[c]
T241	Murine	Fibrosarcoma	−	−	++++[c]
MX-1	Human	Breast carcinoma	−	−	+++[d]
LX-1	Human	Lung carcinoma	−	−	++
CX-1	Human	Colon carcinoma	−	−	+

[a] Degree of reduction: −, none; ±, 0–25%; +, 25–50%; ++, 50–75%; +++, 75–90%; ++++, 90–100%.

[b] 10% complete regressions.

[c] 30% complete regressions.

[d] 40% complete regressions.

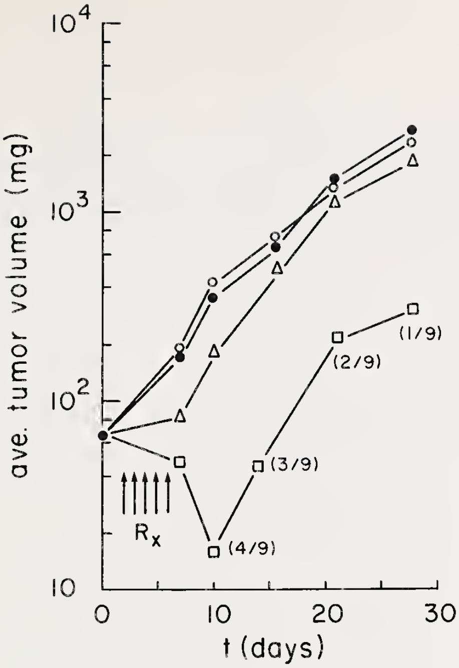


FIGURE 2.—Antitumor activity of MTX, 10dAM and 10EdAM against the MX-1 human mammary adenocarcinoma in nude mice. Analogs were given on a schedule of once per day for 5 days sc starting on day 2 after implantation of tumor. Each point represents the average of 10 animals treated with 3 mg/kg MTX (-○—○-), 1.5 mg/kg 10dAM (-△—△-), or 6 mg/kg 10EdAM (-□—□-), or control animals (-●—●-).

tially without effect. At tolerated doses of 10EdAM (3 mg/kg), tumor growth appeared to be completely inhibited within the period and shortly after therapy with some indication of regression. The overall effect, however, against this tumor was much less than against the other two xenografts.

REFERENCES

(*1*) SIROTNAK FM, CHELLO PL, BROCKMAN RW: Potential for exploitation of transport systems in anticancer drug design. Methods Cancer Res 16:382–447, 1979.

(*2*) SIROTNAK FM, DEGRAW JI: Selective antitumor action of folate analogs. *In* Folate Antagonists as Therapeutic Agents, vol 2. (Sirotnak FM, ed) New York: Academic Press, 1984, pp 43–91.

(*3*) SIROTNAK FM: Correlates of folate analog transport, pharmacokinetics, and selective antitumor action. Pharmacol Ther 8:71–103, 1980.

(*4*) SIROTNAK FM, DEGRAW JI, MOCCIO DM, et al: New folate analogs of the 10-deazaaminopterin series. Basis for structural design and biochemical and pharmacokinetic properties. Cancer Chemother Pharmacol 12:18–25, 1984.

(*5*) SAMUELS LL, MOCCIO DM, SIROTNAK FM: Similar differential for total polyglutamylation and cytotoxicity among various folate analogs in human and murine tumor cells in vitro. Cancer Res 45:1488–1495, 1985.

(*6*) DEGRAW JI, BROWN VH, TAGAWA H, et al: Synthesis and antitumor activity of 10-alkyl,10-deazaaminopterins. A convenient synthesis of 10-deazaaminopterin. J Med Chem 25:1227–1230, 1982.

(*7*) SIROTNAK FM, DEGRAW JI, MOCCIO DM, et al: Antitumor properties of a new folate analog, 10-deazaaminopterin, in mice. Cancer Treat Rep 62:1047–1052, 1978.

(*8*) SIROTNAK FM, DEGRAW JI, CHELLO PL: Biochemical and pharmacologic properties of a new folate analog, 10-deazaaminopterin, in mice. Cancer Treat Rep 66:351–358, 1982.

(*9*) SIROTNAK FM, DEGRAW JI, SCHMID FA, et al: New folate analogs of the 10-deaza-aminopterin series. Further evidence for markedly increased antitumor efficacy compared with methotrexate in ascitic and solid murine tumor models. Cancer Chemother Pharmacol 12:26–30, 1984.

(*10*) SCHMID FA, SIROTNAK FM, OTTER GM, et al: New folate analogs of the 10-deazaaminopterin series: Markedly increased antitumor activity of the 10-ethyl analog compared to the parent compound and methotrexate against some human tumor xenografts in nude mice. Cancer Treat Rep 69:551–553, 1985.

(*11*) GOLDMAN ID: Analysis of the cytotoxic determinants for methotrexate (NSC-740): a role for "free" intracellular drug. Cancer Chemother Rep 6:51–52, 1975.

(*12*) CHABNER BA: Methotrexate. *In* Pharmacologic Principles of Cancer Treatment (Chabner BA, ed). Philadelphia: W.B. Saunders, 1982, pp 229–258.

(*13*) MCQUIRE JJ, MINI E, HSIEH P: Folylpolyglutamate synthetase: relation to methotrexate action and as a target for new drug development. Prog Cancer Res Ther 28:97–106, 1984.

(*14*) GOLDMAN ID, MATHERLY LH, FABRE G: Methotrexate, aminopterin and 7-hydroxymethotrexate: recent concepts in the cellular pharmacology of 4-aminofolates. Prog Cancer Res Ther 28:19–40, 1983.

(*15*) SIROTNAK FM, MOCCIO DM, YANG C-H: Similar characteristics of folate analogue transport in vitro in contrast to varying dihydrofolate reductase levels in epithelial cells at different stages of maturation in mouse small intestine. Cancer Res 44:5204–5211, 1983.

Structural Requirements for the Activity of Antifolates as Substrates for Mammalian Folylpolyglutamate Synthetase[1]

Richard G. Moran,[2,3,]* Paul D. Colman,[2] and Andre Rosowsky[4]

ABSTRACT—**The activity of mouse-liver folylpolyglutamate synthetase (FPGS) was compared using a number of folates and folate analogs in order to determine which structure modifications were compatible with enzyme catalysis and with efficient binding to enzyme. Most structural alterations in the amino acid side chain eliminated activity as a substrate for this enzyme, whereas modifications of any of several positions in the pteridine ring were tolerated with retention of FPGS substrate activity. Folate analogs with the lowest apparent Michaelis constants ($K_{m,app}$) had a) a 4-amino group, b) a 5,6,7,8 reduced ring system, c) a quinazolate ring, and/or d) an unsubstituted 10-position. There was some correlation between FPGS substrate activity and the potency of folate antimetabolites as cytotoxic compounds but not necessarily as compounds selectively cytotoxic to tumor cells.—NCI Monogr 5:133–138, 1987.**

FPGS is an enzyme present in some mammalian tissues (*1,2*) that catalyzes the sequential addition of several moles of glutamic acid to folic acid derivatives. This enzyme system has come under intense investigation in the past few years because of its role in the activation of folate antimetabolites and because it represents a promising "new" target for cancer chemotherapy. FPGS has been purified to homogeneity from 2 bacterial sources (*3,4*), but the isolation of mammalian enzyme has lagged behind due to the low levels of FPGS in mammalian tissues and the lability of the mammalian protein during attempted isolation (*5–9*). However, several salient features of mammalian FPGS have been described. Enzyme from all sources studied to date is present in the soluble fraction of tissues, has an intermediate molecular size (about 65,000 daltons), is somewhat hydrophobic, and is unstable in the absence of ATP and glycerol (*5–8*). Mammalian FPGS can use several nucleotide triphosphates as substrates but has stringent requirements for the structure of the glutamic acid substrate (*7,8,10*). A variety of derivatives of the folic acid molecule can serve as substrates for FPGS, and there are distinct differences in the structures acceptable to mammalian and bacterial enzymes (*3–6,8–11*). Some differences in the structure–activity relationships for substrate activity with mammalian enzyme from different sources have been reported (*11*).

The function of FPGS seems to be the metabolic trapping of folic acid cofactors in forms which are retained inside the cell (*12,13*). Folylmonoglutamates are efficiently transported into and out of mammalian cells by a carrier-mediated system (*14–16*). Folylpolyglutamates of chain length equal to or greater than 3 seem to be poorly accepted by this transport carrier (*17,18*). In addition, the binding of tetrahydrofolate cofactors to enzymes that metabolize these cofactors has most often been found to be substantially more energetically favored for polyglutamate cofactor than for the corresponding monoglutamate derivative [reviewed in (*19*)]. This increase in binding energy associated with polyglutamation of cofactors is not surprising, given the increase in molecular size and the increased anionic character that a polyglutamate side chain lends to the folate cofactors (fig. 1).

Some folate antimetabolites such as methotrexate (MTX) are converted to polyglutamates by FPGS, and the polyglutamate forms of these inhibitors are retained intracellularly under conditions in which the parent compounds are transported to the medium (*20,21*). Polyglutamate forms of MTX have been found to be better inhibitors of several enzymes involved in folate metabolism than MTX (*22,23*). Because the cytotoxic selectivity of antifolates for tumor cells correlates best with cellular retention (*24–26*), it is reasonable that FPGS is involved in the therapeutic activity of antifolates. However, there are probably several factors involved in the selectivity of these compounds, and it remains to be seen whether more easily polyglutamated folate antimetabolites are necessarily more active as chemotherapeutic agents.

FPGS has been demonstrated to be essential for the survival of a growing cell population under normal nutritional conditions (*12*). Somatic cell mutants, in which the enzyme system has been lost, are deficient in the ability to accumulate folates from their environment, presumably as a result of a continued loss of monoglutamates from the cell by efflux (*12*). Hence, it has been suggested that inhibitors of FPGS would have potential as drugs against cancer and other hyperproliferative states (*13*).

In this paper, we examine the specificity with which a partially purified mouse-liver FPGS utilizes folate compounds as substrates by comparing the relative substrate

---

ABBREVIATIONS: FPGS = Folylpolyglutamate synthetase; $K_{m,app}$ = apparent Michaelis constant; MTX = methotrexate; V_{max} = maximal velocity.

---

[1] Supported in part by Grants CA-27605 (RGM) and CA-25394 (AR) from the National Cancer Institute, DHHS.

[2] Division of Hematology/Oncology, Children's Hospital of Los Angeles, and Departments of Pediatrics and Biochemistry, School of Medicine, University of Southern California, Los Angeles, CA.

[3] Scholar of the Leukemia Society of America.

[4] Division of Cancer Pharmacology, Dana-Farber Cancer Institute, Boston, MA.

* *Reprint requests to:* Richard G. Moran, Ph.D., Division of Hematology/Oncology, Children's Hospital of Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027-0700.

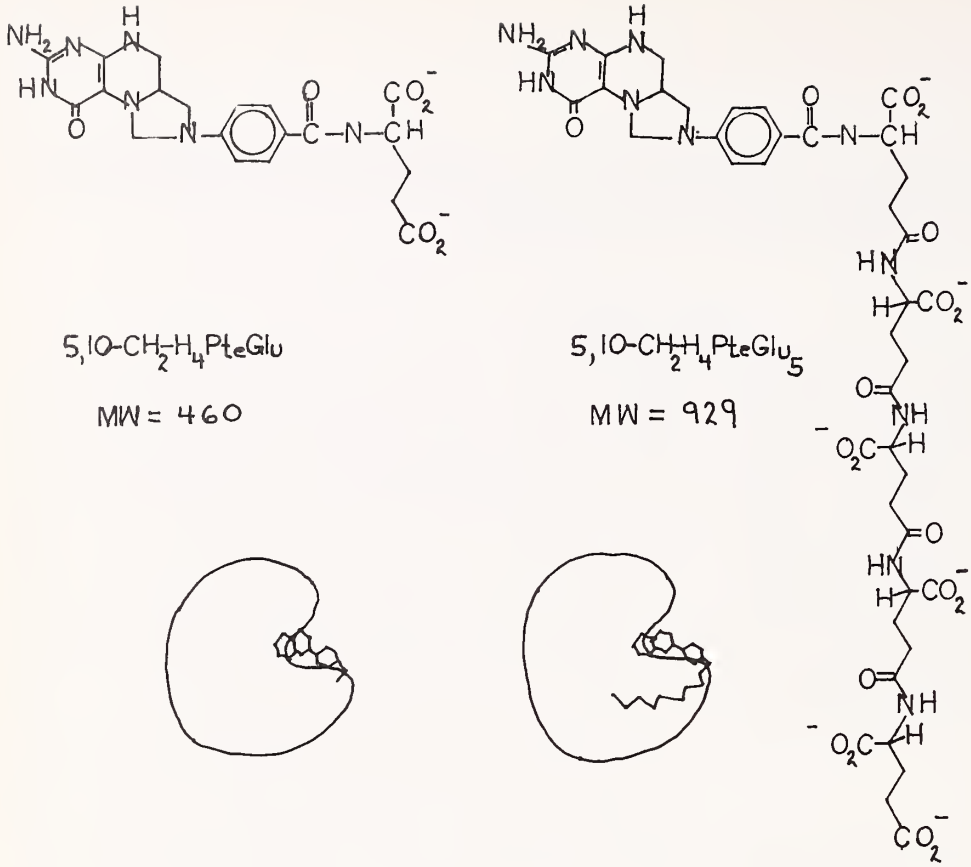


FIGURE 1.—Structures of 5,10-methylenetetrahydropteroylglutamate (*left*) and 5,10-methylenetetrahydropteroylpentaglutamate (*right*). Schematic cartoon at the bottom of this figure contrasts the binding of a folylmonoglutamate into the active site of an enzyme with the binding of a folylpentaglutamate to the same enzyme which involves the binding of several additional ionically charged groups to the protein. MW= molecular weight.

activity of pairs of compounds that differ by a single structural modification. From such an analysis, the contribution of the different regions of the folate molecule to the binding of substrate to the active site of FPGS in a conformation compatible with catalysis can be inferred. Such information would allow, on the one hand, the synthesis or selection of folate analogs that would be unlikely substrates for FPGS and, on the other hand, the structural design of new antifolates with enhanced activity as substrates for polyglutamation.

MATERIALS AND METHODS

The sources of most of the compounds discussed in this work have been described in previous papers (*5,11,27–29*). The purity of compounds used was, in general, determined to be greater than 95% either by DEAE-cellulose chromatography (*30*) and spectral analysis or by reverse-phase high-pressure liquid chromatography (*5*). Folate derivatives that were unstable under aerobic conditions (such as tetrahydrofolic acid and tetrahydroaminopterin) were purified on DEAE-cellulose columns in the presence of 1% 2-mercaptoethanol (*30*) and were studied either immediately after elution from these columns or after storage at −25°C in evacuated ampules. Mouse-liver FPGS was prepared from either DBA/2 or $B6DF_1$ female mice as previously described (*5*) and had a typical specific activity of 1.2 nmol/hour/mg protein. These enzyme preparations were found to be free of interfering conjugase activity under the conditions of our assay (*5*).

The activity of various folates as substrates for this enzyme was determined by incubation of increasing concentrations of compound with enzyme and [^{3}H]glutamic acid for 1 hour followed by immediate isolation of [^{3}H]labelled products by charcoal adsorption (*1*). Previous experiments have demonstrated that the products isolated by this procedure are largely diglutamates as long as short incubation times and low levels of enzyme are used and the extent of consumption of substrate is less than 15%. Hence, this assay measures the conversion of folylmonoglutamate to the corresponding diglutamate if initial reaction rate conditions are maintained. Duplicate reactions were performed at each of at least 6 concentrations of substrate in each experiment and all experiments were performed at least twice. Usually, a control saturation curve was performed in each experiment using folic acid as a substrate to allow normalization of data by the $K_{m,app}$ and maximal velocity (V_{max}) of a standard substrate. This approach was found to decrease variation between the results of replicate experiments. Kinetic parameters were derived using a standard computer program designed for enzyme kinetic analysis (*31*).

RESULTS AND DISCUSSION

Relative Substrate Activity of the Naturally Occurring Folates

A comparison of the kinetics of the FPGS reaction with various naturally occurring folylmonoglutamates revealed that several cofactor forms are excellent substrates and that no 1 compound was preferred. The V_{max} of the FPGS reaction did not vary much among these folylmonoglutamates, but there were major differences in the $K_{m,app}$ within this

TABLE 1.—Substrate activity of the naturally occurring folates for mouse-liver FPGS[a]

Compound	$K_{m, app}$, μM	V_{max}[b]	$V_{max}/K_{m, app}$[b]
Folic acid	137	1.0	1.0
Dihydrofolic acid	8.6	1.57	28
L-Tetrahydrofolic acid	7.0	1.31	30
L-5,-10-Methylenetetrahydrofolic acid	4.8	1.17	26
D,L-5-Formyltetrahydrofolic acid	4.1[c]	0.97	32[c]
D,L-10-Formyltetrahydrofolic acid	2.0[c]	1.25	88[c]
D,L-5-Methyltetrahydrofolic acid	43[c]	1.30	4.0[c]

[a] Data from (*5*).

[b] Relative to PteGlu in the same experiment.

[c] Assuming that only 1 of the diastereoisomers is active.

series (table 1). Enzyme clearly preferred reduced folate cofactors over folic acid. It is interesting that the best substrates of this series were the compounds used as substrates for de novo biosynthesis of purines (i.e., 10-formyltetrahydrofolic acid) and of thymidylate (i.e., 5,10-methylenetetrahydrofolic acid). We have previously noted that structure-activity considerations suggest that the configuration of 5,10-methylenetetrahydrofolic acid seems to favor good fit into the active site of FPGS. This idea is still compatible with all available information. It has been demonstrated that both diastereoisomers about the asymmetric carbon 6 of tetrahydrofolate and of 5-formyltetrahydrofolate are accepted as substrates for mammalian FPGS (*6,32*). However, the naturally occurring diastereoisomer had a significantly lower K_m value than the compound of the opposite orientation, so that the reaction rate of 6-R,S mixtures reflected predominantly the rate of conversion of the natural diastereoisomer (*32*). Hence, the apparent first-order rate constant ($V_{max}/K_{m,app}$) for (6-S) 10-formyltetrahydrofolic acid, the best substrate of the naturally occurring folylmonoglutamates, is probably about 90, relative to that of folic acid.

The data of table 1 demonstrate that a single structural modification can make a major difference in the efficiency with which the folate is accepted as a substrate for FPGS but that almost all of the differences between compounds are due to differences in $K_{m,app}$. It can also be noted that the apparent first order rate constant is very useful as an index of substrate activity.

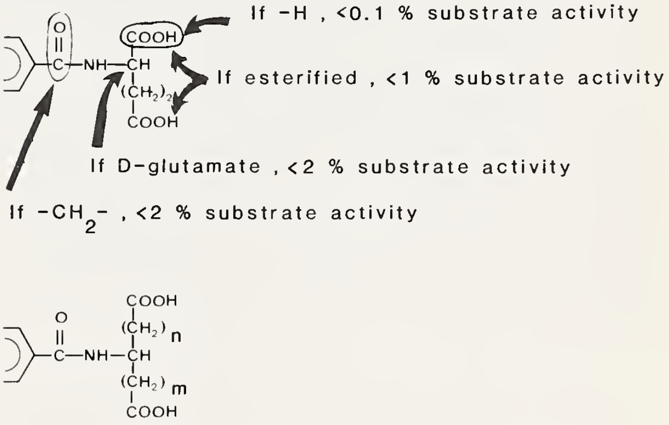
Structural Features That Eliminate Substrate Activity

Mouse-liver FPGS has stringent requirements for the structure of the amino acid side chain. Most deviations from the structure of L-glutamic acid resulted in analogs that are not substrates for polyglutamation (fig. 2). The enzyme was stereospecific for the L-configuration about the 2-carbon in glutamate and required free, unesterified carboxylic acid at both α- and γ-positions. The requirement for a free γ-carboxylic acid group would be expected because of the modification of this position during reaction. It is interesting to note, however, that if a phosphonic acid group or a sulfonic acid group is substituted for the γ-carboxyl of MTX or aminopterin, the resultant analogs are not substrates for FPGS but apparently bind to enzyme as well as the corresponding carboxylic acid compound (*33–35*) (*see* Rosowsky et al. in this symposium). The inactivity of α-esterified analogs of MTX as substrates for FPGS (*11*) could imply either a low bulk-tolerance about this position or a requirement for the α-carboxylic acid group for proper binding to the enzyme. However, the latter explanation seems likely because an MTX analog lacking the α-carboxylic acid (i.e., the γ-aminobutyric acid derivative) is not a substrate for reaction and does not bind well to FPGS (*11,28*). It is also interesting to note that deoxoaminopterin, in which the aromatic carbonyl of aminopterin is replaced by a methylene group (*36*), is essentially inactive as a substrate (*11*).

Mouse-liver FPGS would not tolerate alteration of the distance between the asymmetric carbon of the side chain and the ω-carboxylic acid group without loss of substrate activity (*11*). Derivatives of MTX or aminopterin with one more or one less methylene spacer between the asymmetric carbon and the ω-carboxylic acid than the 2-carbon length of glutamate were completely without activity. An analog of MTX in which the *p*-aminobenzoyl amino group was attached to carbon-3 of glutarate (rather than carbon-2 as in glutamic acid) was also without activity.

Structural Features That Promote Utilization as a Substrate for FPGS at Low Analog Concentrations

The kinetic parameters of the mouse-liver FPGS reaction have been studied (*5,11,27,28*) with enough folate sub-


If –H , <0.1 % substrate activity
If esterified , <1 % substrate activity
If D-glutamate , <2 % substrate activity
If –CH2– , <2 % substrate activity
O
C
NH
CH
COOH
(CH2)2
COOH
(CH2)n
(CH2)m
COOH

n	m	amino acid	substrate activity , %
0	1	L-Aspartate	<1 %
0	2	L-Glutamate	100 %
1	1	3-Aminoglutarate	<1 %
0	3	L-Aminoadipate	1 %
0	4	L-Aminopimelate	21 %
0	5	L-Aminosuberate	1 %

FIGURE 2.—Most structural modifications of the amino acid side chain of folates eliminate activity as a substrate for FPGS. These results were taken from (*11*).

Table 2.—Influence of 5,6,7,8-reduction on substrate activity of folate analogs

Compound	$V_{max}/K_{m, app}$[a]	Ratio[b]
Folic acid	1.0	
L-Tetrahydrofolic acid	30.0	30
Aminopterin	13.8	
D,L-Tetrahydroaminopterin	90.8[c]	6.6
MTX	0.9	
D,L-Tetrahydro-MTX	49.6[c]	55.1
5,10-Dideazafolic acid	1.33	
5,10-Dideazatetrahydrofolic acid	54.2[c]	40.8
5,10-Dideazaaminopterin	0.4	
5,10-Dideazatetrahydro-aminopterin	14.2[c]	35.5
Homofolate	0.54	
L-Tetrahydrohomofolate	1.80	3.3

[a] Relative to folic acid.

[b] Reduced compound/unreduced compound.

[c] Data were obtained on D,L-mixtures but the $V_{max}/K_{m, app}$ values were calculated assuming that only one diastereoisomer was active.

strates of different structures to allow some generalizations to be made about which structural modifications decrease the $K_{m,app}$ of this reaction. Four structural modifications have a major impact on K_m with little effect on V_{max}:

1) Reduction of the 5,6,7,8 system. A number of reduced derivatives had substantially lower $K_{m,app}$ values than did the corresponding unreduced compounds (table 2). This effect was less significant for aminopterin, which was itself an excellent substrate (*11*), and for homofolate, which has an additional methylene group between the pteridine ring and the phenyl ring (*37*). Fully reduced (i.e., 5,6,7,8 tetrahydro-) folate analogs represent the best FPGS substrates as yet described among the antimetabolites.

2) Replacement of the 4-oxo group of the pteridine structure with a 4-amino group. Among the folate analogs studied to date that have a pteridine ring structure and for which an appropriate pair of compounds was available, a 4-amino derivative was a substrate for polyglutamation at much lower concentrations than the corresponding 4-oxo derivative (table 3). However, a limited number of comparisons have been made and this generalization does not hold for at least some analogs with a pyridopyrimidine ring system (table 3).

Table 3.—Effect of replacement of the 4-oxo group with a 4-amino group

Compound	$V_{max}/K_{m, app}$[a]	Ratio[b]
Folic acid	1.0	
Aminopterin (4-Deoxy-4-aminofolic acid)	13.3	13.3
10-Methylfolic acid	0.045	
MTX	0.90	20.0
Tetrahydrofolic acid	30.0	
Tetrahydroaminopterin	90.8	3.0
5,10-Dideazafolic acid	1.33	
5,10-Dideazaaminopterin	0.40	0.30
5,10-Dideazatetrahydrofolic acid	54.2	
5,10-Dideazatetrahydro-aminopterin	14.2	0.26

[a] Relative to folic acid.

[b] Reduced compound/unreduced compound.

Table 4.—Effect of structural changes at the 10-position of the folate molecule

Compound	$V_{max}/K_{m, app}$[a]	Ratio[b]
Alkylation		
Folic acid	1.0	
10-CH_3-folic acid	0.05	0.05
Aminopterin	13.3	
10-Methylaminopterin (MTX)	0.90	0.068
Substitution		
Folic acid	1.0	
10-Thiofolic acid	1.1	1.1
Aminopterin	13.3	
10-Deaza-aminopterin	0.95	0.071

[a] Relative to folic acid.

[b] Reduced compound/unreduced compound.

3) Derivatives with a quinazoline ring system. These seem to be better substrates than the corresponding pteridine ring-containing analogs.

4) An unsubstituted 10-nitrogen. This confers superior FPGS activity. A comparison of a small number of either 4-oxo or 4-amino folates with methylated and free 10-nitrogens suggests that alkylation of this position diminishes the efficiency of binding of folates to FPGS by a substantial factor (table 4). Near-isosteric replacement of the 10-nitrogen with sulfur was without effect. However, if the 10-nitrogen was replaced with a methylene group, the ability of the resultant compound (10-deazaaminopterin) to serve as a substrate was diminished.

Correlation of Efficiency of Polyglutamation of Folate Antimetabolites and Therapeutic Activity

Several folate antimetabolites have been made within the past few years that either inhibit targets other than dihydrofolate reductase or potentially offer more selectivity than MTX. Many if not all of these compounds that have the structure of "classical"[5] antifolates are substrates for FPGS (table 5) and, therefore, would be expected to be polyglutamated in tumor cells in vivo and possibly in normal tissues. The antitumor and enzyme inhibitory potency of some of these therapeutically relevant compounds is correlated with their activity as substrates for mouse-liver FPGS in table 5. Several speculations are prompted by this comparison: 1) The potency of antitumor activity and of host toxicity seems to correlate with the efficiency of activation by FPGS. That is, among folate antimetabolites inhibitory to either dihydrofolate reductase (MTX, aminopterin, dichloromethotrexate) or purine de novo synthesis (5,10-dideazatetrahydrofolate, 5-methyltetrahydrohomofolate, 5-formyltetrahydrohomofolate) (*38–40*), compounds that are better substrates of FPGS are active at lower dosages. Whether such a relationship exists for folate inhibitors of thymidylate synthetase cannot be determined because of

---

[5] That is, that contain a ring system similar to a pteridine nucleus linked, via a *p*-aminobezoyl bridge, to a glutamic acid moiety.

TABLE 5.—Antitumor potency of some folate antimetabolites and their substrate activity for FPGS

Compound	$K_{m, app}$	Comments
10-Formyltetrahydrofolic acid	88[a]	Best folylmonoglutamate substrate. Cofactor for purine synthesis.
Tetrahydroaminopterin	91[a]	Moderate inhibitor of TS[b] in vitro. Unstable in oxygenated solution.
5,10-Dideazatetrahydrofolic acid	54[a]	Most potent folate inhibitor of de novo purine synthesis known.
Tetrahydromethotrexate	50[a]	Moderate inhibitor of TS in vitro. Unstable in oxygenated solution.
Aminopterin	13	Exceptionally potent inhibitor of DHFR. Active in vivo at much lower doses than MTX.
Dichloromethotrexate	4	Potent inhibitor of DHFR.
CB 3717	2	Most potent folate inhibitor of TS known.
10-Deazaaminopterin	1.0	Potent inhibitor of DHFR.
MTX	0.9	Potent inhibitor of DHFR in vitro. Much less potent than aminopterin in vivo.
7-Hydroxymethotrexate	0.7	
5-Methyltetrahydrohomofolate	0.4	Inhibitor of purine synthesis in culture. Very nontoxic in phase 1 trial.
5-Formyltetrahydrohomofolate	0.1	Inhibitor of purine synthesis. Much less potent than 5,10-dideazatetrahydrofolic acid.
4-Amino-10-methylfolylhomocysteic acid	0.0	Strong inhibitor of DHFR in vitro. Active in vivo only with frequent administration.

[a] Assuming that only one diastereoisomer is active or that the activity of the one far outweighs that of the other.

[b] TS=Thymidylate synthetase.

the paucity of such inhibitors that are active in vivo. It should be noted that this apparent relationship could be due to either enhanced intracellular retention or enhanced binding of polyglutamated inhibitor to target enzyme(s) or both factors.

2) The dosage regimen that allows optimal activity of an inhibitor may be different for compounds of equal activity against a target enzyme but of dissimilar potential for polyglutamation. Thus, strong inhibitors of dihydrofolate reductase that are not substrates for FPGS (e.g., 4-amino-10-methylpteroylcysteic acid) are more active if given frequently than if given intermittently (*34*). MTX, a moderate FPGS substrate, has been found to be more active on an intermittent schedule. Aminopterin, an excellent substrate for FPGS, has been previously found to be more toxic than MTX when both are compared on the same intermittent schedule and to have less of a therapeutic ratio than MTX when the two are compared on the same schedule.

3) There is little or no information available at present that suggests that activity as a substrate for FPGS predicts for selective toxicity against tumor cells. Much more work is needed to address this potential correlation.

REFERENCES

(*1*) MORAN RG, COLMAN PD: Measurement of folylpolyglutamate synthetase in mammalian tissues. Anal Biochem 140:326–342, 1984.

(*2*) MCGUIRE JJ, KITAMOTO Y, HSIEH P, et al: Characterization of mammalian folylpolyglutamate synthetases. *In* Chemistry and Biology of Pteridines (Kisliuk RL, Brown GM, eds). New York: Elsevier/North Holland Press, 1979, pp 471–476.

(*3*) SHANE B: Pteroylpoly(γ-glutamate) synthesis by *Corynebacterium* species: Purification and properties of folylpoly(γ-glutamate) synthetase. J Biol Chem 255:5655–5662, 1982.

(*4*) BOGNAR AL, SHANE B: Purification and properties of *Lactobacillus casei* folylpoly-γ-glutamate synthetase. J Biol Chem 258:12574–12581, 1983.

(*5*) MORAN RG, COLMAN PD: Mammalian folylpolyglutamate synthetase: Partial purification and properties of the mouse liver enzyme. Biochemistry 23:4580–4589, 1984.

(*6*) MCGUIRE JJ, HSIEH P, COWARD JK, et al: Enzymatic synthesis of folylpolyglutamates: Characterization of the reaction and its products. J Biol Chem 255:5776–5788, 1980.

(*7*) TAYLOR RT, HANNA ML: Folate-dependent enzymes in cultured Chinese hamster cells: Folylpolyglutamate synthetase and its absence in mutants auxotrophic for glycine + adenosine + thymidine. Arch Biochem Biophys 181: 331–334, 1977.

(*8*) CICHOWICZ D, COOK J, GEORGE S, et al: Hog liver folylpolyglutamate synthetase: Substrate specificity and regulation. *In* Proceedings of The Second Workshop on Folyl and Antifolyl Polyglutamates (Goldman ID, ed). New York: Praeger Press, 1985, pp 7–13.

(*9*) SCHOO MM, PRISTUPA ZB, VICKERS PJ, et al: Folate analogues as substrates of mammalian folylpolyglutamate synthetase. Cancer Res 45:3034–3041, 1985.

(*10*) CICHOWICZ D, FOO SK, SHANE B: Folylpoly-γ-glutamate synthesis by bacteria and mammalian cells. Mol Cell Biochem 39:209–228, 1981.

(*11*) MORAN RG, COLMAN PD, ROSOWSKY A, et al: Structural features of 4-amino antifolates required for substrate activity with mammalian folylpolyglutamate synthetase. Mol Pharmacol 27:156–166, 1985.

(*12*) MCBURNEY MW, WHITMORE GF: Isolation and biochemical characterization of folate-deficient mutants of Chinese hamster cells. Cell 2:173–182, 1974.

(*13*) MORAN RG: Characterization of the function of mammalian folate polyglutamate synthetase. *In* Folyl and Antifolyl Polyglutamates (Bertino JR, Chabner BA, Goldman ID, eds). New York: Plenum Press, 1983, pp 327–339.

(*14*) LICHTENSTEIN NS, OLIVERIO VT, GOLDMAN ID: Characteristics of folic acid transport in the L1210 leukemia cell. Biochim Biophys Acta 193:456–467, 1969.

(*15*) NAHAS A, NIXON PF, BERTINO JR: Uptake and metabolism of N^5-formyltetrahydrofolate by L1210 leukemia cells. Cancer Res 32:1416–1421, 1972.

(*16*) Goldman ID: The characteristics of the membrane transport of amethopterin and the naturally occurring folates. Ann NY Acad Sci 186:400–422, 1969.

(*17*) Baugh CM, Krumdieck CL, Baker HJ, et al: Folate absorption in the dog after exposure of isolated intestinal segments to synthetic pteroylpolyglutamates of various chain lengths. J Clin Invest 50:2009–2021, 1971.

(*18*) Godwin HA, Rosenberg IH: Comparative studies of the intestinal absorption of pteroylmonoglutamate and pteroylheptaglutamate. Gastroenterology 69:364–373, 1975.

(*19*) McGuire JJ, Bertino JR: Enzymatic synthesis and function of folylpolyglutamates. Mol Cell Biochem 38:19-48, 1981.

(*20*) Fry DW, Yalowich JC, Goldman ID: Rapid formation of poly-γ-glutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by HPLC in the Ehrlich ascites carcinoma cell. J Biol Chem 257:1890–1896, 1982.

(*21*) Galivan J: Evidence for the cytotoxic activity of polyglutamate derivatives of methotrexate. Mol Pharmacol 17:105–110, 1980.

(*22*) Allegra CJ, Drake JC, Jolivet J, et al: Inhibition of phosphoribosylaminoimidazolcarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. Proc Natl Acad Sci USA 82:4881–4885, 1985.

(*23*) Allegra CJ, Chabner BA, Drake JC, et al: Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. J Biol Chem 260:9720–9726, 1985.

(*24*) Sirotnak FM: Role of membrane transport in the pharmacologic action of antifolates. Cancer Treat Rep 65:19–27, 1981.

(*25*) Sirotnak FM, Donsbach RC: Differential cell permeability and the basis for selective activity of methotrexate during therapy of the L1210 leukemia. Cancer Res 33:1290–1294, 1973.

(*26*) Sirotnak FM, Donsbach RC: Comparative studies on the transport of aminopterin, methotrexate and methasquin by the L1210 leukemia cell. Cancer Res 32:2120–2126, 1972.

(*27*) Taylor EC, Harrington PJ, Fletcher SR, et al: Synthesis of the antileukemic agents 5,10-dideazaaminopterin and 5,10-dideaza-5,6,7,8-tetrahydroaminopterin. J Med Chem 28:914–921, 1985.

(*28*) Moran RG, Colman PD, Forsch RA, et al: A mechanism for the addition of multiple moles of glutamate by folylpolyglutamate synthetase. J Med Chem 27:1263–1267, 1984.

(*29*) Rosowsky A, Forsch RA, Uren J, et al: Synthesis and antitumor activity of the α-aminoadipic, α-aminopimelic, and α-aminosuberic acid side chain analogues of methotrexate. J Med Chem 26:1719–1724, 1983.

(*30*) Moran RG, Zakrzewski SF, Werkheiser WC: Folate metabolism in mammalian cells in culture: Partial characterization of the folates present in L1210 mouse leukemia cells. J Biol Chem 25:3569–3575, 1976.

(*31*) Cleland WW: The statistical analysis of enzyme kinetic data. Adv Enzymol 29:1–32, 1967.

(*32*) Sato JA, Moran RG: Interaction of methotrexate and citrovorum factor (CF) at folylpolyglutamate synthetase. Proc Am Assoc Cancer Res 25:312, 1984.

(*33*) Rosowsky A, Moran RG, Forsch R, et al: Methotrexate analogues. 17. The antitumor activity of 4-amino-4-deoxy-N^{10}-methylpteroyl-D,L-homocysteic acid and its dual inhibition of dihydrofolate reductase and folylpolyglutamate synthetase. Biochem Pharmacol 33:155–161, 1984.

(*34*) Rosowsky A, Forsch RA, Freisheim JH, et al: Methotrexate analogs: 19. Replacement of the glutamate side-chain in classical antifolates by L-homocysteic acid and L-cysteic acid: Effect on enzyme inhibition and antitumor activity. J Med Chem 27:600–604, 1984.

(*35*) Rosowsky A, Moran RG, Forsch R, et al: Methotrexate Analogues. 27. Dual inhibition of dihydrofolate reductase and folylpolyglutamate synthetase by methotrexate and aminopterin analogues with a γ-phosphonate group in the side chain. Biochem Pharmacol 35:3327–3333, 1986.

(*36*) Rosowsky A, Forsch R: Methotrexate analogues. 16. Importance of the side chain amide carbonyl group as a structural determinant of biological activity. J Med Chem 25:1454–1459, 1982.

(*37*) Goodman L, DeGraw J, Kisliuk RL, et al: Tetrahydrohomofolate: A specific inhibitor of thymidylate synthetase. J Am Chem Soc 86:308–309, 1964.

(*38*) Hakala MT: Homofolate and tetrahydrohomofolate, inhibitors of purine synthesis. Cancer Res 31:813–816, 1971.

(*39*) Moran RG, Taylor EC, Beardsley GP: 5,10-Dideaza-5,6,7,8-tetrahydrofolic acid (DATHF): A potent antifolate inhibitory to de novo purine synthesis. Proc Am Assoc Cancer Res 26:231, 1985.

(*40*) Moran RG, Rosowsky A, Forsch R: A new folate antimetabolite specifically inhibitory to de novo purine synthesis. Proc Am Assoc Cancer Res 25:311, 1984.

Folate Analog Nonsubstrates and Inhibitors of Folylpolyglutamate Synthetase as Potential Cancer Chemotherapy Drugs[1]

John J. McGuire,[2,3,*] **James R. Piper,**[4] **James K. Coward,**[5] **and John Galivan**[6,7]

ABSTRACT—Folate monoglutamates and classical antifols such as methotrexate (MTX) are converted intracellularly to poly-γ-glutamyl forms by folylpolyglutamate synthetase (FPGS). Folylpolyglutamates are absolutely essential for cell survival and proliferation. MTX polyglutamates are strongly implicated in the cytotoxic mechanism of this drug. Two new types of antifol could be targeted toward polyglutamate synthesis. One type would be structurally analogous to a folate monoglutamate or MTX, except it would not form polyglutamates. In the case of a folate, this analog might induce cellular deficiency by displacing natural folates. In the case of MTX, the analog might have a therapeutic advantage if MTX polyglutamates are involved primarily in host toxicity. The second approach is through direct inhibitors of FPGS. Two relevant MTX analogs have been synthesized and tested: 4-amino-10-methylpteroyl-4-fluoroglutamate does not form polyglutamates or does so extremely poorly; the second, 4-amino-10-methylpteroyl-2,5-diaminopentanoate, is an inhibitor of mammalian FPGS, as predicted from the work of Shane and co-workers with reduced pteroyl-2,5-diaminopentanoate.—NCI Monogr 5:139-144, 1987.

Natural folate monoglutamates are converted intracellularly to poly-γ-glutamyl forms. A characteristic distribution of lengths is formed which is organism specific (*1*). These polyglutamyl derivatives are synthesized by the enzyme folylpolyglutamate synthetase (FPGS) according to the general equation:

$$\mathrm{PteGlu_N + ATP + L\text{-}Glu ---> PteGlu_{N+1} + ADP + P_i},$$

where $\mathrm{PteGlu_N}$ = polyglutamated derivatives of folic acid in which N = total glutamates in γ-linkage, and P_i = inorganic phosphate. A number of functions are now known for the folylpolyglutamates [reviewed in (*2,3*)]. The most general functions are: (a) folylpolyglutamates serve as the actual intracellular cofactors and inhibitors in one-carbon metabolism, often having higher ratios of maximum velocity (V_{max}) to Michaelis-Menten constant (K_m), or lower inhibitory constants (K_i) than the corresponding monoglutamates, and (b) folylpolyglutamates allow cellular retention and concentration of folates because of their polyanionic nature. Regardless of function, the observation that all organisms synthesize folylpolyglutamates suggests they are important. A second observation suggests they are, in fact, essential. Deletion of folylpolyglutamate synthesis is a lethal mutation (*4*) which allows cells to grow only in the presence of all end products of folate metabolism; that is, only when folate metabolism is unnecessary. Even very high extracellular reduced folate levels do not allow growth, indicating that monoglutamates are unable to fulfill some essential function(s).

Methotrexate (MTX) is also known to be converted to poly-γ-glutamyl derivatives in tissues and cultured cells. As with folylpolyglutamates, the MTX polyglutamates are retained by cells and allow intracellular drug concentrations far in excess of that in the extracellular space to be maintained. Although MTX polyglutamates may only bind to dihydrofolate reductase (DHFR), the target enzyme, as tightly as does MTX, they can bind to and inhibit other folate-dependent enzymes with affinities up to 2,500-fold greater than MTX itself. Thus, secondary targets of MTX may be inhibited only by polyglutamate forms. A large body of work has firmly implicated MTX polyglutamates in the cytotoxic mechanism of this drug, especially during short exposure periods [reviewed in (*5*)]. There is some evidence that polyglutamylation is a determinant in the selectivity of this drug against malignant as compared to normal tissues.

The essential requirement for folylpolyglutamates for viability and the importance of MTX polyglutamates in tumor cytotoxicity suggests that alterations in polyglutamate synthesis could have profound therapeutic effects. Alterations in polyglutamate synthesis can be arrived at indirectly (by changes in the one-carbon distribution in the folate pool, for example) or directly through effects on FPGS. In the latter regard, our studies (*6–12*), and those of others (*13–15*), on the structure-function relationships for FPGS substrates have been useful in developing compounds with modified properties with respect to polyglu-

---

ABBREVIATIONS: EC_{50} = drug concentration inhibiting growth by 50%; FGlu = 4-fluoroglutamate; FPGS = folylpolyglutamate synthetase; FMTX = γ-fluoro-MTX(4-amino-10-methyl-pteroyl[4-fluoroglutamate]); I_{50} = concentration required for 50% inhibition; K_i = inhibitory constant; K_{is} = slope inhibitory constant; K_m = Michaelis-Menten constant; MTX = methotrexate; P_i = inorganic phosphate; PteGlu = folic acid; $\mathrm{PteGlu_N}$ = polyglutamate derivatives of folic acid in which the subscript "N" = total glutamates in γ-linkage; V_{max} = maximum velocity.

[1] Supported in part by grants CA-08010, CA-25933, CA-28097, and CA-25236 from the National Cancer Institute, Public Health Service, Department of Health and Human Services, and grant CH-288 from the American Cancer Society.

[2] Department of Pharmacology, Yale University School of Medicine, New Haven, CT.

[3] Scholar of the Leukemia Society of America.

[4] Drug Synthesis Section, Southern Research Institute, Birmingham, AL.

[5] Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY.

[6] New York State Department of Health, Albany, NY.

[7] We thank A. R. Cashmore and W. Whyte-Bauer for supplying purified dihydrofolate reductases, P. Hsieh for technical assistance, and Judith Barger for help in preparing this manuscript.

* *Reprint requests to:* John J. McGuire, Ph.D., Grace Cancer Drug Center, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263.

tamylation. Based on these studies, we have used 2 distinct approaches to the design of antifols targeted toward polyglutamate synthesis. The first approach is the design of antifols which directly interact with and inhibit FPGS. The second approach is the design of compounds which are equivalent to classical antifols and folates in all properties except they are nonsubstrates for FPGS and thus do not form polyglutamates.

MATERIALS AND METHODS

Analogs of MTX in which the glutamic acid residue was replaced by a homologous series of 2,ω-diaminoalkanoic acids (*16*) or with 4-fluoroglutamic acid (*17*) were prepared as described. Purified DHFR was obtained from two human leukemia cell lines (K562 and CCRF–CEM) and assayed spectrophotometrically (*18*). The concentrations required for 50% inhibition (I_{50}) for DHFR inhibitors were obtained from graphs of the logarithm of drug concentration versus activity. K562 and CCRF-CEM human leukemia cell FPGS were prepared by fractionating freeze–thaw lysates (in 0.1 *M* Tris-HCl, pH 8.85; 0.1 *M* 2-mercaptoethanol) with $(NH_4)_2SO_4$ (0–40% saturation pellet) followed by size exclusion (BioGel A–0.5 *M*) chromatography. The active fractions (in 20 m*M* K-phosphate, pH 7.5 with 500 m*M* KCl and 50 m*M* 2-mercaptoethanol) were concentrated, taken to 20% (vol/vol) glycerol, and stored at −90° C. FPGS was assayed under standard conditions with L-[^{3}H]glutamate as a substrate (*6,8*) and DEAE–cellulose minicolumns were used to separate free [^{3}H]glutamate from that ligated to a folate substrate. Kinetic data were obtained over a 10-fold concentration range in the presence or absence of inhibitor. Radiolabel incorporation was demonstrated to be linear with respect to time under all conditions. Standard methods were used to analyze kinetic data and obtain kinetic constants (*19*). Since FPGS gives multiple products readily with some monoglutamate substrates (e.g., tetrahydrofolate), kinetic studies were performed only with aminopterin which gives essentially only the diglutamate during the incubation period under standard conditions (unpublished observation). Thus, Michaelis-Menten kinetics were maintained. Rabbit liver aldehyde oxidase was purified 46-fold according to our modification (*10*) of a published procedure (*20*). Aldehyde oxidase was assayed at room temperature by measuring the change in absorbance at 341 nm as MTX (or its analog) was converted to its 7-hydroxylated derivative (*20*). Transport studies were performed by a micromethod utilizing repeated chilled saline washes to remove extracellular drug (*21*). Incorporation of [^{3}H]deoxyuridine (Moravek Biochemicals) into DNA was measured by collecting cells exposed to radiolabel on glass fiber filters that were then washed with 20 ml of 3% TCA (0° C), followed by 10 ml of 95% ethanol before being dried for liquid scintillation counting (*4*).

Cells were cultured using standard sterile technique. The myeloblastic leukemia cell line K562 (*22*) was grown in RPMI 1640 (GIBCO) containing 10% filtered fetal bovine serum (GIBCO). The lymphoblastic cell line CCRF-CEM(*23*) was cultured in RPMI 1640 containing 10% filtered horse serum (GIBCO). A subline of CCRF-CEM, resistant to MTX because of defective MTX transport (*24*) was cultured in the same medium containing 1 *μM* MTX. These cells were grown in the absence of MTX for 48 hours prior to their use in cytotoxicity studies.

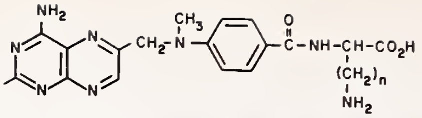


FIGURE 1.—Structure of MTX analogs containing 2,ω-diaminoalkanoic acids instead of L-glutamate. Analogs containing n = 1–3 were tested. The ornithine-containing derivative is *n* = 3.

To measure drug concentrations which inhibited growth by 50% (EC_{50}), logarithmically growing cells were seeded at 1×10^4 cells/ml in the absence or presence of various concentrations of drug. Following 120 hours of continuous exposure to the drug, a cell count was performed using a model ZB1 Coulter counter. Control samples (containing no drug) grew logarithmically during this entire period. The EC_{50} values were obtained from plots of logarithm drug concentration versus percent of control growth.

RESULTS AND DISCUSSION

Effects of Methotrexate Analogs Containing 2,ω-Diaminoalkanoic Acids

In the search for direct inhibitors of FPGS, attention has been focused on folate analogs, since the widespread metabolism of ATP and glutamate would probably render their analogs nonspecific. However, FPGS exhibits strict structural specificity (*3*). Thus, most folate analogs are either substrates or simply do not bind and are not inhibitors. Recently, Shane's group (*13*) reported that reduced folates containing ornithine (2,5-diaminopentanoic acid), instead of L-glutamate, were potent ($K_i = 0.2$ *μM*) inhibitors of hog liver FPGS. Based on this observation, we have investigated a homologous series (fig. 1) of MTX analogs containing 2,ω-diaminoalkanoic acids, including ornithine, as inhibitors of DHFR, FPGS, and cell growth.

The 2,ω-diaminoalkanoic acid containing analogs of MTX were potent inhibitors of 2 human DHFR, although none was as potent as MTX itself (table 1). Lymphoblastic and myeloid DHFR were nearly identical in their sensitivity to each compound. As the length of the side chain of the analogs increased, the DHFR inhibitory potency also increased.

TABLE 1.—Inhibition of dihydrofolate reductase and folylpolyglutamate synthetase by 2,ω-diaminoalkanoic acid analogs of MTX[a]

Enzyme source	Inhibitor 4-amino-10-methylpteroyl-R R=	DHFR inhibition, ID_{50}, n*M*	FPGS inhibition, K_{is}, *μM*[b]
K562	2,5-diaminopentanoate	4	4.1
	2,4-diaminobutanoate	7.5	110
	2,3-diaminopropanoate	18	>2000
	glutamate (MTX)	1	
CCRF-CEM	2,5-diaminopentanoate	2.5	3.2
	2,4-diaminobutanoate	5.7	330
	2,3-diaminopropanoate	17	
	glutamate (MTX)	1	

[a] Compounds tested as described in Materials and Methods.

[b] K_{is} values were obtained from replots of slope (derived from double reciprocal plots) versus inhibitor concentration.

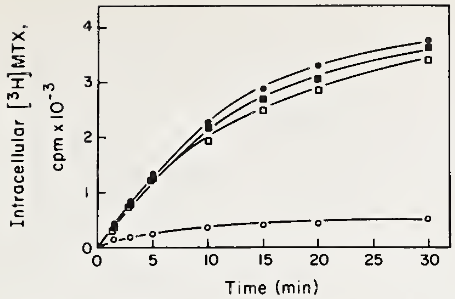


FIGURE 2.—Inhibition of [^{3}H]MTX transport into CCRF-CEM cells by the 2,5-diaminopentanoate containing analog of MTX. CCRF-CEM cells (1.2×10^6/ml) were harvested by centrifugation (5 min; 1000×*g*) and resuspended at 2.1×10^7/ml in RPMI 1640 containing 10% horse serum and 20 m*M* HEPES–NaOH, pH 7.5. Cells were equilibrated for 5 min at 37°C, and inhibitor was added to appropriate cultures for an additional 5 min (preincubation). [^{3}H]MTX (3 μCi/ml; 2 μM final concentration) was added. Samples were removed at the appropriate time and processed as described in Materials and Methods. Conditions during the preincubation were: control, no addition (●); 20 μM (■) or 40 μM (□) of the 2,5-diaminopentanoate derivative; 20 μM aminopterin (○).

All the 2,ω-diaminoalkanoic acid containing analogs of MTX lacked substrate activity with both human leukemia cell FPGS. However, each analog was inhibitory to FPGS, indicating that binding was still occurring (table 1). The inhibitory potency again increased as the chain length increased, but the effect was more dramatic than with DHFR. The 2,5-diaminopentanoate (ornithine) derivative was most potent with a slope inhibitory constant (K_{is}) near 4 μM for both lymphoblastoid and myeloid FPGS. Inhibition by all derivatives was linear competitive. A high-pressure liquid chromatographic analysis of the products synthesized by isolated FPGS (data not shown) showed a decline in all products that was dependent on inhibitor concentration. Thus, each step in elongation appeared to be sensitive to inhibition, although part of the reduction in each product might be attributable to the decrease in its precursor(s).

MTX and other 2,4-diamino antifols are transported into mammalian cells by a carrier-mediated process which normally transports reduced folates and, possibly, PteGlu (*25*). The 2,5-diaminopentanoate analog of MTX was tested as an inhibitor of [^{3}H]MTX uptake in CCRF-CEM cells to determine if it, too, used the same carrier (fig. 2). Aminopterin was used as a positive control to show that MTX influx could be effectively inhibited. The results show that even at a 20-fold excess, the analog caused little inhibition of [^{3}H]MTX influx. Thus, either MTX and its analog do not share a common influx carrier or the affinity of the analog for this common carrier is extremely poor.

MTX is metabolized in significant amounts by aldehyde oxidase to its 7-hydroxylated form in man (*26*). This is an inactivation reaction in that 7-hydroxy-MTX is a much less potent inhibitor of DHFR than is MTX (*10*). The aldehyde oxidase substrate activity of MTX and its 2,5-diaminopentanoate analog were compared (fig. 3). The K_m of the analog (9 μM) was strikingly lower than that of MTX (164 μM), but its V_{max} was only 22% that of MTX. The product of both reactions was identical to that of authentic 7-hydroxy-MTX.

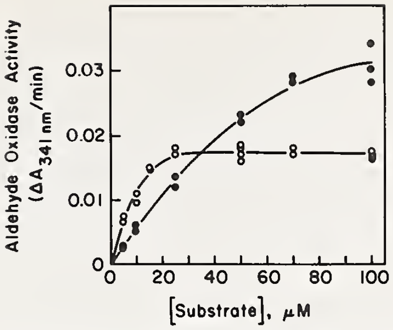


FIGURE 3.—Concentration dependence of rabbit liver aldehyde oxidase substrate activity of MTX and 4-amino-10-methylpteroyl (2,5-diaminopentanoic acid). Partially purified rabbit-liver aldehyde oxidase was assayed as described in Materials and Methods with either methotrexate (●) or 4-amino-10-methylpteroyl(2,5-diaminopentanoate) (○) as the substrate.

With the myeloid K562 cell line there were small but reproducible differences in cytotoxicity of the 3 analogs that contained 2,ω-diaminoalkanoate groups (table 2). The 2,5-diaminopentanoate derivative was the most potent, but it was still 100-fold less cytotoxic than MTX. Because inactivation of the pentanoate derivative by aldehyde oxidase occurred much more readily than did inactivation of MTX, it seemed possible that other mechanisms of inactivation might be similarly increased. To rule this out as the reason for the difference in cytotoxicity, we determined the drug levels in medium containing cells at time zero and at the end of a cytotoxicity incubation (120 hr). The drug was completely stable during this period.

The CCRF-CEM lymphoblastoid cell line was 70-fold less sensitive to the pentanoate derivative than to MTX (table 2). However, a subline of CCRF-CEM that is 250-fold resistant to MTX because of defective MTX transport is only 5-fold less sensitive to the 2,5-diaminopentanoate derivative than is the parental line (table 2).

In summary, a series of MTX analogs containing 2,ω-diaminoalkanoic acids instead of glutamate has been investigated. The most cytotoxic compound, containing 2,5-diaminoalkanoate (ornithine) instead of L-glutamate, may have two sites of action since it is inhibitory to both isolated DHFR and FPGS. It is presently unclear why an amino

TABLE 2.—Cytotoxic effects on K562 and CCRF-CEM human leukemia cell lines of 2,ω-diaminoalkanoic acid analogs of MTX[a]

Cell line	Inhibitor 4-amino-10-methylpteroyl-R R=	EC_{50}, μM
K562	2,5-diaminopentanoate	1.7
	2,4-diaminopentanoate	2.3
	2,3-diaminopropanoate	3.0
	glutamate (MTX)	0.0175
CCRF-CEM	2,5-diaminopentanoate	0.74
	glutamate (MTX)	0.011
CCRF-CEM(MTX^R)[b]	2,5-diaminopentanoate	3.7
	glutamate (MTX)	2.7

[a] Cytotoxicity was measured by outgrowth following a 120-hr continuous exposure to inhibitor according to procedures in Materials and Methods.

[b] Cell line resistant to MTX by virtue of defective MTX transport (*24*).

function replacing the γ-carboxyl should allow such strong binding to FPGS. Although the ornithine-containing compound is less cytotoxic than MTX itself, it has the intriguing property that MTX transport-defective resistant cells are only minimally cross-resistant to this analog. Further investigation of this analog may allow more specific and more potent inhibitors of FPGS to be designed. The altered substrate activity of such analogs with aldehyde oxidase must be kept in mind during use of such analogs in vivo, however.

Effects of Methotrexate Analogs Containing 4-Fluoroglutamic Acid

A determination of the biological effects of polyglutamates could be made if a suitable analog could be synthesized with properties identical to the parent compound, except for being a nonsubstrate for FPGS. Such compounds have been made possible by recent work on the specificity of FPGS for addition of free glutamic acid. We showed (*11*) that when 4-fluoroglutamate (FGlu) replaced glutamate in the reaction, only 1 FGlu was ligated to the folate substrate. These studies demonstrated that FGlu was an alternate substrate for FPGS and acted as a chain terminator. This result suggested to us that if the glutamate present on the folate substrate was replaced by FGlu (e.g., pteroyl-FGlu) the resultant analog would form polyglutamates poorly or not at all. However, the isosteric replacement of hydrogen by fluorine might not alter the other properties of the molecule. These FGlu-containing analogs could thus be used to define the roles and importance of polyglutamates of the Glu-containing derivative under a variety of different conditions.

The first such analog prepared (*17*) was γ-fluoromethotrexate [4-amino-10-methylpteroyl(4-fluoroglutamate); FMTX], the FGlu-containing analog of MTX (fig. 4). Providing the only substantive difference in properties between the analog and MTX itself is the FPGS substrate activity, it should be possible to use this analog in two types of studies. First, the importance of MTX polyglutamates in cytotoxicity could be measured by comparing the effect of MTX and FMTX. Second, the importance of MTX polyglutamates in the efficacy or specificity of MTX treatment could be measured in vivo. Only the first question has been addressed so far.

The properties of FMTX were examined first; the inhibition of DHFR was of primary interest. The I_{50} values for FMTX against the DHFR from H-35 hepatoma cells (0.96 μM) and human leukemic spleen (0.95 μM) were found to be similar to the values for MTX (0.88 and 0.75 μM, respectively). Since thymidylate synthase is often mentioned as a secondary target for MTX (*27*), we also examined inhibition of this enzyme. FMTX and MTX gave nearly identical weak inhibition of thymidylate synthase from the CCRF-CEM human leukemia cell line ($I_{50} = 0.21$ and 0.23 mM, respectively). Intracellular accumulation of FMTX or MTX was determined using a DHFR inhibition assay and the H-35 rat hepatoma system (*17*). The results showed that the intracellular accumulation of each drug between 30 and 120 minutes at 5 μM extracellular concentration was nearly equivalent, although the initial uptake of MTX was somewhat more rapid. Efflux of FMTX and unmetabolized MTX from H-35 cells occurred at the same rapid rate and both effluxed until the cellular level of DHFR was reached (*17*). To this point the properties of MTX and FMTX seem very similar. However, their properties as substrates for FPGS are quite different (table 3). FMTX displays little substrate activity for FPGS while activity is high when MTX is the substrate. This low activity is a result of poor binding of FMTX to FPGS because it is only a very weak inhibitor of FPGS activity when MTX is used as the substrate (table 3). Thus, FMTX fulfills the criteria for a compound useful in studying roles of polyglutamate, i.e., it has all the properties of its congener MTX, except it cannot form polyglutamates.

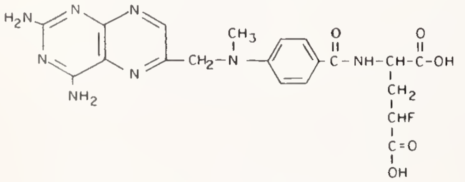


FIGURE 4.—The structure of FMTX.

TABLE 3.—Substrate and inhibitor activity of FMTX with FPGS[a]

MTX, μM	FMTX, μM	Total glutamate incorporated, pmol	Relative activity, %[b]
100	—	5070, 4490	—
25	—	2320, 2330	100
—	100	190, 60	5
25	100[c]	2020, 2030	87

[a] FPGS was isolated from rat liver and the partially purified enzyme was assayed as described in Materials and Methods.

[b] Based on average values of the duplicates shown.

[c] FMTX concentration is 4 times that of MTX, since only the L-threo diastereoisomer of FMTX is presumed to act as a substrate [(*15*); *see* text].

Two approaches were used in comparing the effects of MTX and FMTX. First, direct cytotoxicity measurements were made using CCRF-CEM cells and the H-35 rat hepatoma either continuously or for short periods of time following which drug was removed. With both cell lines (table 4) the toxicity of FMTX was less than that of MTX even

TABLE 4.—Effect of exposure time on growth inhibition of H-35 and CCRF-CEM cells by MTX and FMTX[a]

Exposure time, hr	EC_{50}, μM		FMTX/MTX (ratio of EC_{50})
	MTX	FMTX	
H-35 rat hepatoma			
2	0.3	700	2300
4	0.1	34	340
7	0.06	10	167
24	0.02	0.4	20
72	0.01	0.12	12
CCRF-CEM human leukemia			
4	0.21	ND[b]	
7	0.08	7.5	114
24	0.02	0.4	20
120	0.004	0.076	19

[a] CCRF-CEM cells were exposed to drug for the indicated times, following which drug was removed by washing. Cell counts were all performed at 120 hr as described in Materials and Methods. Studies with H-35 cells were performed as previously described (*17*).

[b] Not determined.

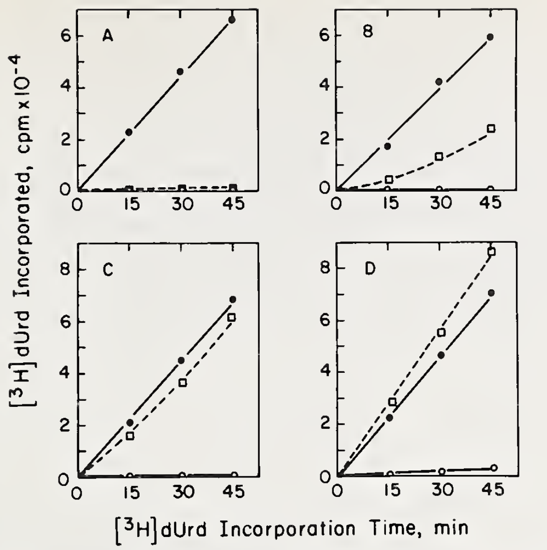


FIGURE 5.—Inhibition of incorporation of [^{3}H]deoxyuridine ([^{3}H]dUrd) into DNA of CCRF-CEM cells by MTX and FMTX. Logarithmically grown CCRF-CEM cells (6.7×10^5/ml) were harvested by centrifugation ($1000\times g$) at 37° C and resuspended in RPMI 1640 containing 10% horse serum and 20 m*M* HEPES-NaOH, pH7.5 at 7.7×10^5 cells/ml. Aliquots (22 ml) were incubated at 37° C for 4 hr with: no drug, control (●); 2 μM MTX (○); or 2 μM FMTX (□). After 4 hr, an aliquot of each condition was removed to a new vessel and [^{3}H]deoxyuridine (0.5 μCi/ml) was added. Incorporation into DNA was measured at the indicated times over 45 min as described in Materials and Methods (A). The remainder of the cells were centrifuged at 37° C, washed thoroughly, and resuspended at the same cell density in the above medium, lacking drug. At appropriate intervals (efflux time) after resuspension, aliquots were removed and [^{3}H]deoxyuridine incorporation was measured as described in Materials and Methods. (B) 1-hr efflux. (C) 2-hr efflux. (D) 4-hr efflux.

under continuous exposure conditions. This difference could result from the slightly weaker inhibition of DHFR observed with FMTX, or the fact that the FMTX used is a mixture of 4 isomers (*17*), not all of which may be active, or that polyglutamates of MTX contribute to cytotoxicity even under continuous exposure conditions. This problem is currently under investigation. As the exposure time was decreased the disparity in growth inhibition between MTX and FMTX increased dramatically (table 4). This is especially clear for the hepatoma cells where a 2-hour exposure was tested, and a 2300-fold difference in cytotoxicity was found. With the hepatoma cells, the reduction from 72-hour to 2-hour exposure caused only a 30-fold decrease in toxicity for MTX, but the toxicity of FMTX decreased by 6000-fold. The lower toxicity of FMTX following short exposure to FMTX suggests that folate metabolism and DNA synthesis are not inhibited as well in these cells or both recover faster in drug-free medium. These possibilities were investigated in the CCRF-CEM cells using a 4-hour exposure to MTX or FMTX and incorporation of [^{3}H]-deoxyuridine into DNA. The results (fig. 5A) show that after 4 hours, [^{3}H]deoxyuridine incorporation is inhibited completely by either 2 μM MTX or 2 μM FMTX. One hour after drug was removed, the MTX-treated cells are still completely inhibited while the FMTX-treated cells are significantly recovered (fig. 5B). By 2 hours after drug removal, FMTX-treated cells have returned nearly to control levels of [^{3}H]deoxyuridine incorporation and the MTX-treated cells are still completely inhibited (fig. 5C). After 4 hours in drug-free medium, the FMTX-treated cells exhibit the characteristic "overshoot" incorporation while MTX-treated cells are only beginning to recover (fig. 5D). Thus, it seems that the lower cytotoxicity of FMTX is a result of faster recovery of DNA synthesis by these cells when placed in drug-free medium. Since the only significant difference between MTX and FMTX is their ability to be polyglutamylated, it follows that their vastly different cytotoxicites and abilities to inhibit DNA synthesis must result from this difference. Thus the evidence strongly suggests that toxicity of MTX is strongly dependent on synthesis of polyglutamates, especially during brief exposure times.

REFERENCES

(*1*) COSSINS EA: Folates in biological materials. *In* Chemistry and Biochemistry of Folates (Blakely RL, Benkovic SJ, eds). New York: John Wiley and Sons, 1984, pp 1–59.

(*2*) MCGUIRE JJ, BERTINO JR: Enzymatic synthesis and function of folylpolyglutamates. Mol Cell Biochem 38:19–48, 1981.

(*3*) MCGUIRE JJ, COWARD JK: Pteroylpolyglutamates. Biosynthesis, degradation, and function. *In* Chemistry and Biochemistry of Folates (Blakely RL, Benkovic SJ, eds). New York: John Wiley and Sons, 1984, pp 135–190.

(*4*) MCBURNEY MW, WHITMORE GF: Isolation and biochemical characterization of folate deficient mutants of chinese hamster cells. Cell 2:173–182, 1974.

(*5*) CHABNER BA, ALLEGRA CJ, CURT GA, et al: Polyglutamation of methotrexate. Is methotrexate a prodrug? J Clin Invest 76:907–912, 1985.

(*6*) MCGUIRE JJ, HSIEH P, COWARD JK, et al: Enzymatic synthesis of folylpolyglutamates. Characterization of the reaction and its products. J Biol Chem 255:5776–5788, 1980.

(*7*) MCGUIRE JJ, HSIEH P, COWARD JK, et al: Studies on the substrate specificity of mammalian folylpolyglutamate synthetase. *In* Proceedings of the Sixth International Symposium on Chemistry and Biology of Pteridines (Blair JA, ed). Berlin: de Gruyter, 1983, pp 615–619.

(*8*) MCGUIRE JJ, HSIEH P, COWARD JK, et al: In vitro methotrexate polyglutamate synthesis by rat liver folylpolyglutamate synthetase and inhibition by bromosulfophthalein. Adv Exptl Med Biol 163:199–214, 1983.

(*9*) MCGUIRE JJ, MINI E, HSIEH P, et al: Folylpolyglutamate synthetase: relation to methotrexate action and as a target for new drug development. *In* The Development of Target Oriented Anticancer Drugs (Chen Y-C, Gaz B, Minkoff M, eds). New York: Raven Press, 1983, pp 97–106.

(*10*) MCGUIRE JJ, HSIEH P, BERTINO JR: Enzymatic synthesis of polyglutamate derivatives of 7-hydroxymethotrexate. Biochem Pharmacol 33:1355–1361, 1984.

(*11*) MCGUIRE JJ, COWARD JK: DL-Threo-4-fluoroglutamic acid. A chain-terminating inhibitor of folylpolyglutamate synthesis. J Biol Chem 260:6747–6754, 1985.

(*12*) MCGUIRE JJ, HSIEH P, FRANCO CT, et al: Folylpolyglutamate synthetase inhibition and cytotoxic effects of methotrexate analogs containing 2,ω-diaminoalkanoic acids. Biochem Pharmacol 35:2607–2613, 1986.

(*13*) CICHOWICZ D, COOK J, GEORGE S, et al: Hog liver folylpolyglutamate synthetase: Substrate specificity and regulation. *In* Proceedings of the Second Workshop on Folyl and Antifolylpolyglutamates (Goldman ID, ed). New York: Praeger, 1985, pp 7–13.

(*14*) MORAN RG, COLMAN PD, ROSOWSKY A, et al: Structural features of 4-amino antifolates required for substrate activity with mammalian folylpolyglutamate synthetase. Mol Pharmacol 27:156–166, 1985.

(*15*) Schoo MMJ, Pristupa ZB, Vickers PJ, et al: Folate analogues as substrates of mammalian folylpolyglutamate synthetase. Cancer Res 45:3034–3041, 1985.

(*16*) Piper JR, McCaleb GS, Montgomery JA, et al: Syntheses and evaluation as antifolates of MTX analogues derived from 2,ω-diaminoalkanoic acids. J Med Chem 28:1016–1025, 1985.

(*17*) Galivan J, Inglese J, McGuire JJ, et al: Gamma-fluoromethotrexate: Synthesis and biological activity of a potent inhibitor of dihydrofolate reductase with greatly diminished ability to form poly-gamma-glutamates. Proc Natl Acad Sci USA 82:2598–2602, 1985.

(*18*) Osborn MJ, Huennekens FM: Enzymatic reduction of dihydrofolic acid. J Biol Chem 233:969–974, 1958.

(*19*) Segel IH: Enzyme Kinetics. New York: Wiley Interscience, 1975.

(*20*) Johns DG, Iannotti AJ, Sartorelli AC, et al: The relative toxicities of methotrexate and aminopterin. Biochem Pharmacol 15:555–561, 1966.

(*21*) Kamen BA, Cashmore AR, Dreyer RN, et al: Effect of [^{3}H]methotrexate impurities on apparent transport of methotrexate by a sensitive and resistant L1210 cell line. J Biol Chem 255:3254–3257, 1980.

(*22*) Lozzio CB, Lozzio BB: Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. Blood 45:321–334, 1975.

(*23*) Foley GF, Lazarus H, Farber S, et al: Continuous culture of lymphoblasts from peripheral blood of a child with acute leukemia. Cancer 18:522–529, 1965.

(*24*) Rosowsky A, Lazarus H, Yuan GC, et al: Effects of methotrexate esters and other lipophilic antifolates on methotrexate-resistant human leukemic lymphoblasts. Biochem Pharmacol 29:648–652, 1980.

(*25*) Sirotnak FM: Correlates of folate analog transport, pharmacokinetics and selective antitumor action. Pharmacol Ther 8:71–103, 1980.

(*26*) Chan KK, Nayar MSB, Cohen JL, et al: Metabolism of methotrexate in man after high and conventional doses. Res Commun Chem Pathol Pharmacol 28:551–561, 1980.

(*27*) Borsa J, Whitmore GF: Studies relating to the mode of action of methotrexate. III. Inhibition of thymidylate synthetase in tissue culture cells and in cell-free systems. Mol Pharmacol 5:318–332, 1969.

Synthesis and Biologic Activity of New Side-Chain-Altered Methotrexate and Aminopterin Analogs With Dual Inhibitory Action Against Dihydrofolate Reductase and Folylpolyglutamate Synthetase[1]

Andre Rosowsky,[2,*] Richard G. Moran,[3,4] James H. Freisheim,[5] Henry Bader,[2] Ronald A. Forsch,[2] and Vishnu C. Solan[2,6]

ABSTRACT—Replacement of the glutamic acid (Glu) moiety in methotrexate (MTX) and aminopterin (AMT) by 2-amino-4-phosphonobutyric acid (APBA) and ornithine (Orn) has been found to give analogs that retain the ability to inhibit dihydrofolate reductase (DHFR) while also displaying high activity against folylpolyglutamate synthetase (FPGS). One of these compounds, the Orn analog of AMT, is the most potent FPGS inhibitor we have found to date. A model to account for the fact that side-chain analogs containing a basic and those containing an acidic terminal group can both competitively inhibit FPGS is proposed. According to this model, binding may involve interaction of an acidic terminal group on the inhibitor with a positively charged active-site residue to which the γ-carboxyl of the folate-antifolate substrate normally binds. It may also involve the interaction of a basic terminal group on the inhibitor with a different active-site residue which is negatively rather than positively charged and to which the α-amino group of the incoming Glu cosubstrate must bind before an amide bond to the γ-carboxyl of the folate-antifolate can form. The 2 oppositely charged active-site residues assumed to take part in this binding are probably situated near each other and at approximately the same distance from the pteridine-binding site. The ability of compounds to inhibit both DHFR and FPGS makes it possible in principle for such compounds to kill cells via a "self-potentiation" mechanism in which inhibition of tetrahydrofolate synthesis is complemented by interference with the subsequent conversion of tetrahydrofolates to their polyglutamate conjugates. Possible exploitation of this mechanism to overcome MTX resistance is considered.—NCI Monogr 5:145-152, 1987.

MTX has been among the world's most widely prescribed anticancer drugs for more than 3 decades and continues to be the object of intensive clinical and laboratory investigation (*1*). Despite the major advances these investigations have produced in our knowledge of the biochemical and pharmacological interactions affecting the outcome of MTX chemotherapy, there remain unanswered questions about precisely how MTX kills cells and why patients with certain types of tumors either fail to respond at all to this drug or cease to respond after an initially successful period of treatment. Systematic modification of the molecular structure of MTX is one of the important approaches available to investigators in the antifolate field as a means of gaining greater knowledge about the biochemical sites of action of MTX and about the mechanism of MTX resistance. A comprehensive historical account of this area of antifolate research has appeared recently (*2*).

We have been engaged for some time in systematic modification of the "γ-terminal region" of MTX with a view to obtaining analogs with favorably altered biochemical and/or pharmacological properties, including especially the ability to overcome MTX resistance. Among the analog types previously reported by us in this connection may be mentioned γ-monoesters (*3-10*), chain-extended compounds with extra CH_2 spacers (*11,12*), and compounds in which COOH is replaced by SO_3H (*13,14*). The sulfonic acid analogs of MTX and of the closely related classical antifolate, AMT, were of unusual interest because they inhibited not only DHFR, the classical target of MTX and AMT, but also FPGS. We postulated that such dual inhibitors might be acting via "self-potentiation", in that inhibition of the reduction of dihydrofolate to tetrahydrofolate by DHFR could be reinforced by concomitant inhibition of the conversion of tetrahydrofolate cofactors from the monoglutamate to the polyglutamate form. If this concept could be reduced to practice, it would represent an interesting new strategy for antifolate drug design.

While MTX and AMT γ-sulfonates displayed a moderate level of inhibitory activity in FPGS assays (*13,14*), their inhibitory constants (K_i) were not low enough relative to

---

ABBREVIATIONS: AMT = aminopterin; APBA = 2-amino-4-phosphonobutyric acid; BOC = *tert*-butyloxycarbonyl; DEPC = diethyl phosphorocyanidate; DHFR = dihydrofolate reductase; [E] = enzyme; FPGS = folylpolyglutamate synthetase; Glu = glutamic acid; HCysA = homocysteic acid; IC_{50} = 50% inhibitory concentration; K_i = inhibitory constant; K_m = substrate constant; mAPA = 4-amino-4-deoxy-N^{10}-methylpteroic acid; MTX = methotrexate; Orn = ornithine.

---

[1] This work was supported in part by Grants CA-25394 (AR), CA-27605 (RGM), CA-39867 (RGM, AR), and CA-41461 (JHF) from the National Cancer Institute, Department of Health and Human Services.

[2] Dana-Farber Cancer Institute and the Department of Pharmacology, Harvard Medical School, Boston, MA.

[3] Division of Hematology/Oncology, Children's Hospital of Los Angeles, CA.

[4] Scholar of the Leukemia Society of America.

[5] Department of Biochemistry, Medical College of Ohio, Toledo.

[6] We thank P. Colman and M. Radike-Smith for technical assistance in obtaining enzyme and cell-growth inhibition data.

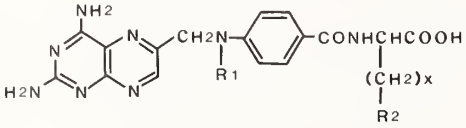
* *Reprint requests to:* Andre Rosowsky, Ph.D., Division of Cancer Pharmacology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

the substrate constant (K_m) for reduced folates to allow the self-potentiation idea to be adequately tested. This paper describes 2 other γ-modifications involving replacement of the Glu side-chain with APBA and Orn, respectively (*15,16*). This has produced compounds with considerably enhanced anti-FPGS activity. One of these, the Orn analog of AMT, is presently the most potent inhibitor we have available, with a K_i of 0.15 μM for the murine enzyme. The finding that replacement of COOH by CH_2NH_2 in the γ-terminal region could bring about such strong FPGS inhibition was surprising but reinforced the prior report by Cichowicz and coworkers (*17*) that the corresponding tetrahydrofolyl derivative is a strong inhibitor. These compounds may thus be an important lead in our search for other, still more potent inhibitors of FPGS.

CHEMISTRY

The chemical structures of the MTX and AMT analogs discussed in this paper are shown in figure 1. It should be noted that the APBA analogs we have studied at this point are racemic (D,L), whereas the Orn analogs are the L-enantiomers.

The syntheses of mAPA-D,L-APBA and APA-D,L-APBA are presented in figure 2. 4-Amino-4-deoxy-N^{10}-methylpteroic acid (mAPA) (*18,19*) was condensed with methyl-D,L-2-amino-4-phosphonobutyrate with the aid of the peptide bond-forming reagent diethyl phosphorocyanidate (DEPC) (*20*) in *N,N*-dimethylformamide solution containing a tertiary amine. The methyl ester of D,L-APBA was obtained by reaction with methanol in the presence of thionyl chloride. The utility of DEPC for the preparation of mAPA derivatives has been demonstrated by us previously (*7,8,11–14,18*). The COOH group in the *p*-methylaminobenzoyl moiety of mAPA is less reactive than the COOH in typical aliphatic amino acids, and therefore either fails to give desired products or gives poor yields when more conventional coupling methods are used (*21,22*). Removal of the methyl ester from the DEPC coupling product was achieved by mild saponification with dilute base. For the synthesis of APA-D,L-APBA, 4-amino-4-deoxy-N^{10}-formylpteroic acid (*8*) was condensed with methyl-D,L-2-amino-4-phosphonobutyrate by a special modification of the mixed carboxylic carbonic anhydride method involving sequential addition of activating reagent (isobutyl chloroformate), tertiary amine, and amino ester in several cycles until all the starting material is consumed (*8,13,14*). Removal of the methyl ester and N^{10}-formyl blocking groups to form APA-D,L-APBA was accomplished in a single step by mild treatment with sodium hydroxide. Cleavage of the methyl ester was very rapid, while removal of the N^{10}-formyl group required somewhat more time. The products, mAPA-D,L-APBA and APA-D,L-APBA, were purified by ion-exchange chromatography on DEAE-cellulose with 3% ammonium bicarbonate as the eluent and were isolated as hydrated hemiammonium salts by freeze-drying.



	R1	x	R2
MTX analogues			
mAPA-D,L-APBA	CH_3	2	PO_3H_2
mAPA-L-Orn	CH_3	3	NH_2
AMT analogues			
APA-D,L-APBA	H	2	PO_3H_2
APA-L-Orn	H	3	NH_2

FIGURE 1.—Chemical structures of APBA and Orn analogs of MTX and AMT.

The syntheses of the Orn analogs, mAPA-L-Orn and APA-L-Orn, are depicted in figure 3. Our synthesis of mAPA-L-Orn differs from one reported recently by other workers (*23*) in that it involves coupling of a protected L-Orn derivative with mAPA. The L-Orn side-chain was introduced in this instance via the *p*-nitrophenyl ester method. The *p*-nitrophenyl esters of mAPA and 4-amino-4-deoxy-N^{10}-formylpteroic acid were obtained by reaction of the acids with *bis*(*p*-nitrophenyl) carbonate (*8*). L-Orn was used as its N^{δ}-*tert*-butyloxycarbonyl (BOC) derivative, and the carboxyl group was either protected by trimethylsilylation or was left unprotected. When the carboxyl group was not protected, an extra equivalent of tertiary amine was added to the reaction. Yields of the condensation products were comparable to those typically obtained via DEPC or mixed anhydride coupling when based on the amount of amino acid used. An advantage of the *p*-nitrophenyl ester method is that it can be carried out with equimolar amounts of reactants, whereas DEPC and mixed anhydride coupling requires at least a 2-fold molar excess of the amino acid derivative. Removal of the N^{10}-formyl group in the AMT analog was accomplished with dilute sodium hydroxide at room temperature, and cleavage of the N^{δ}-BOC protecting group to form mAPA-L-Orn and APA-L-Orn was performed with trifluoroacetic acid at low temperature. mAPA-L-Orn was purified by ion-exchange chromatography on sulfoethoxycellulose with 0.1 *N* ammonium bicarbonate as the eluent; APA-L-Orn was purified more easily, by merely reprecipitating it from aqueous acetic acid with ammonia.

DHFR INHIBITION

The D,L-APBA and L-Orn analogs of MTX and AMT were tested as inhibitors of DHFR purified by affinity chromatography from L1210/R71 or L1210/R81 cells as previously described (*24,25*). These MTX-resistant cell lines are a convenient source of DHFR and the enzyme is similar to the one from wild-type L1210 cells in terms of its kinetics of dihydrofolate reduction and its susceptibility to inhibition by MTX. The IC_{50} of each compound as an inhibitor of dihydrofolate reduction is shown in table 1, along with comparison data for MTX, AMT, and the D,L-homocysteic acid (D,L-HCysA) analogs studied earlier (*13,14*).

The binding affinities of the γ-phosphonates, as measured by IC_{50}, differed only slightly from those of the D,L-γ-

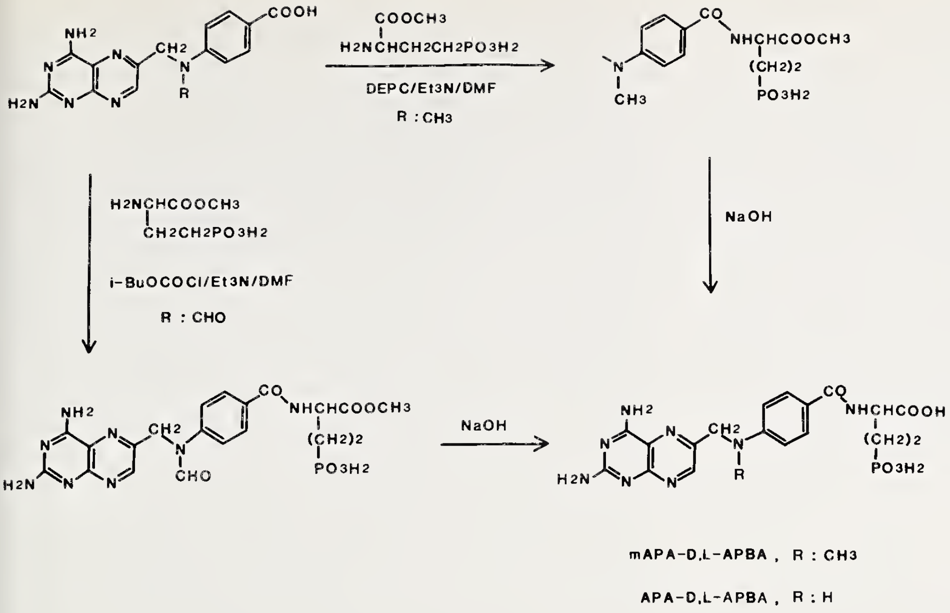


Figure 2.—Chemical syntheses of APBA analogs of MTX and AMT.

sulfonates or those of the parent drugs. However it may be noted that the data for the γ-phosphonates were obtained with D,L-racemates rather than with pure L-enantiomers. The D-enantiomer of MTX has been reported to be a very poor inhibitor in comparison with the L-enantiomer (*26*), and one might therefore expect the L-APBA derivatives to have an IC_{50} lower than those of the D,L-APBA derivatives.

An interesting difference was observed in the ratio of IC_{50} to enzyme ([E]) concentration for the γ-phosphonate and γ-sulfonate analogs. In addition to normalizing the data for differences in enzyme concentration among different experiments, the $IC_{50}/[E]$ ratio appears to be an indicator of deviation from linearity in the enzyme titration curve at high inhibitor concentrations. While the ratio for the parent compounds and γ-sulfonates was 0.5–0.6, the ratio for the γ-phosphonates was approximately twice as great, indicating greater deviation from linearity for the latter compounds. A possible explanation of this effect is that the γ-phosphonic acid group forms a dianion rather than a monoanion at pH 7.5. The extra negative charge on the end of the side-chain may result in an increased rate of dissociation ("off rate") (*27*) of the inhibitor from the enzyme active site. Deviation from linearity with increasing inhibitor concentration is important insofar as it means that 2 compounds with almost the same IC_{50} for DHFR inhibition may nonetheless differ substantially in their ability to lower tetrahydrofolate pools to the point where thymidylate biosynthesis is completely suppressed (*28*).

The IC_{50} of mAPA-L-Orn as a DHFR inhibitor was 0.160 μM and its $IC_{50}/[E]$ ratio was 2.3. The corresponding

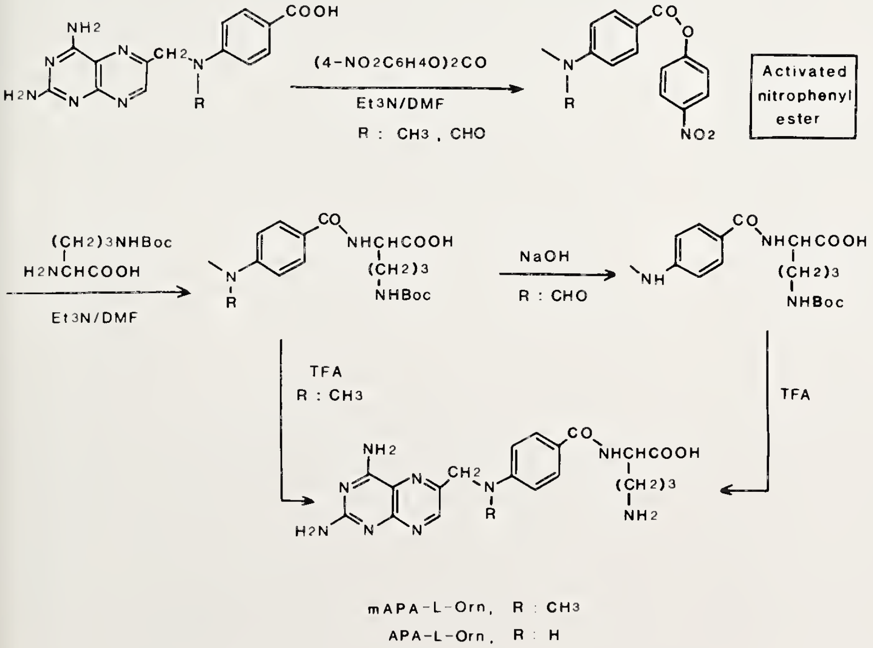


Figure 3.—Syntheses of Orn analogs of MTX and AMT.

TABLE 1.—Inhibition of DHFR from L1210 murine leukemia cells by HCysA, APBA, and Orn analogs of MTX and AMT

Compound	IC_{50}, μM[a]	$IC_{50}/[E]$
MTX	0.050	0.50
mAPA-D,L-HCysA	0.055	0.55
mAPA-D,L-APBA	0.063	1.3
mAPA-L-Orn	0.160	2.3
AMT	0.040	0.50
APA-D,L-HCysA	0.062	0.62
APA-D,L-APBA	0.067	1.3
APA-L-Orn	0.072	1.0

[a] DHFR was purified by affinity chromatography as previously described (*25*). The assay mixture contained 0.04–0.1 μM enzyme and 75 μM NADPH in 10 mM Tris, pH 7.2, or 50 mM Tris, pH 7.5. After 2 min of preincubation at 22°C, the reaction was started by adding 50 μM dihydrofolate. The reaction was followed spectrophotometrically at 340 nm (*25*).

values for APA-L-Orn were 0.072 μM and 1.0. Comparison of the $IC_{50}/[E]$ ratios for these compounds with those for MTX and AMT revealed a 4.6-fold decrease in inhibitory potency toward DHFR upon replacement of the Glu side-chain by Orn in MTX, whereas the same modification in AMT produced only a 2-fold decrease in potency. The relatively high $IC_{50}/[E]$ ratio for mAPA-L-Orn and APA-L-Orn in comparison with MTX and AMT was of interest because it suggested that a positive charge on the end of the amino acid side-chain had an adverse effect on DHFR binding. The fact that this effect was more pronounced for the N^{10}-methyl than the N^{10}-unsubstituted derivative was also of interest, as it suggested that N^{10}-substitution, apart from altering the torsional angle of the phenyl ring, may actually also induce a change in orientation of the more distant amino acid side-chain, including even the γ-terminal region, in the enzyme-bound form of the drug.

FPGS INHIBITION

In addition to being tested as DHFR inhibitors, the D,L-APBA and L-Orn analogs of MTX and AMT were assayed as inhibitors of mouse liver FPGS activity (*13,29,30*). Assays were performed at pH 8.6 with folic acid as the variable substrate. A short reaction time (1 hr) and low amount of enzyme were used to ensure that the results would predominantly reflect addition of a single glutamate residue. Conjugase activity was negligible for this partially purified enzyme preparation, especially under the alkaline pH conditions of the assay (*31*). K_i were calculated from the slopes of double-reciprocal plots. Competitive reciprocal plots in the presence and absence of inhibitor intersected the vertical axis at a common point. K_i values for the D,L-APBA and L-Orn analogs, as well as for the corresponding D,L-HCysA derivatives for comparison, are presented in table 2. The K_i for the γ-phosphonate and γ-sulfonate analogs of MTX were essentially the same, and did not differ much from the Michaelis-Menten constant (K_m) for folic acid. The K_i/K_m ratio for these compounds was therefore close to one. The K_i for mAPA-D,L-HCysA and mAPA-D,L-APBA were also comparable to the reported K_m of 166±49 μM for MTX (*29*) with mouse liver FPGS. This suggested that binding of these analogs to the FPGS active site occurs readily but is nonproductive for polyglutamylation. By analogy with the data for AMT and APA-D,L-HCysA in comparison with MTX and mAPA-D,L-HCysA, we were expecting that the K_i for APA-D,L-APBA would be approximately 1/4 the K_i for mAPA-D,L-APBA. The K_i for APA-D,L-APBA proved, however, to be 1/20 the K_i for mAPA-D,L-APBA, giving a K_i/K_m ratio of 0.06 as compared with 1.3 for the N^{10}-methyl derivative. Moreover the K_i for APA-D,L-APBA as an inhibitor was 2-fold lower than the reported substrate K_m of 17.6±5.8 μM for AMT (*29*). These results suggested that, where binding to murine FPGS is concerned, AMT may be more sensitive than MTX to changes involving replacement of the γ-COOH by other acidic groups. It is not immediately clear why there should be such a difference between N^{10}-methyl and N^{10}-unsubstituted compounds, but it is of interest that the same pattern was also observed with respect to DHFR inhibition (see above).

The results in table 2 also reveal the surprising fact that replacement of the γ-COOH in MTX and AMT by a basic CH_2NH_2 group produces compounds that are even better than the γ-sulfonate or γ-phosphonate analogs as inhibitors of mouse-liver FPGS. The K_i of mAPA-L-Orn was 9-fold lower than that of either mAPA-D,L-HCysA or mAPA-D,L-APBA, while the K_i of APA-D,L-Orn was 300-fold lower than that of APA-D,L-HCysA and 56-fold lower than that of APA-D,L-APBA. We recently reported that APA-L-Orn and APA-D,L-APBA also inhibit FPGS in extracts of a human colon carcinoma line grown in immunosuppressed mice, and that the K_i of these compounds against the human enzyme is several times lower than their K_i against murine enzyme (*16*). APA-L-Orn is the most potent competitive inhibitor of mammalian FPGS that we have uncovered thus far in the intensive search for such inhibitors that we and others have been conducting for several years (*17,29,32–36*). Moreover, the K_i for inhibition by this compound is in a range (<0.2 μM) that should be attainable in cells under physiological conditions.

The activity shown by the compounds in table 2 is noteworthy in the context of recent work on γ-fluoromethotrexate, which is reported to be a potent DHFR inhibitor but a poor FPGS substrate (*36*). In the presence

TABLE 2.—Inhibition of mouse liver FPGS by HCysA, APBA, and Orn analogs of MTX and AMT

Compound	K_i, μM[a]	K_i/K_m
mAPA-D,L-HCysA	188±56	1.72±0.46
mAPA-D,L-APBA	185±71	1.20±0.17
mAPA-L-Orn	20.4±7.7	0.091±0.034
APA-D,L-HCysA	45±6.0	0.32±0.025
APA-D,L-APBA	8.4±2.0	0.052±0.017
APA-L-Orn	0.15±0.06	0.00089±0.00019

[a] The enzyme was obtained by precipitation from cell lysates with 30% ammonium sulfate, and typically had a specific activity of 1.2 nmol/hr/mg protein. The assay mixture contained 10–500 μM folic acid, 1 mM [^{3}H]L-glutamic acid, 5 mM ATP, 10 mM $MgCl_2$, 30 mM KCl, 20 mM 2-mercaptoethanol, and 20 mM Tris, pH 8.6 in a volume of 0.25 ml. Incubation was for 1 hr. Conjugase activity was negligible under these conditions. K_i/K_m ratios were calculated by using the apparent Michaelis constant ($K_{m,app}$) for folic acid measured in each experiment. This value was 140±47 μM in a typical series of experiments (*29*).

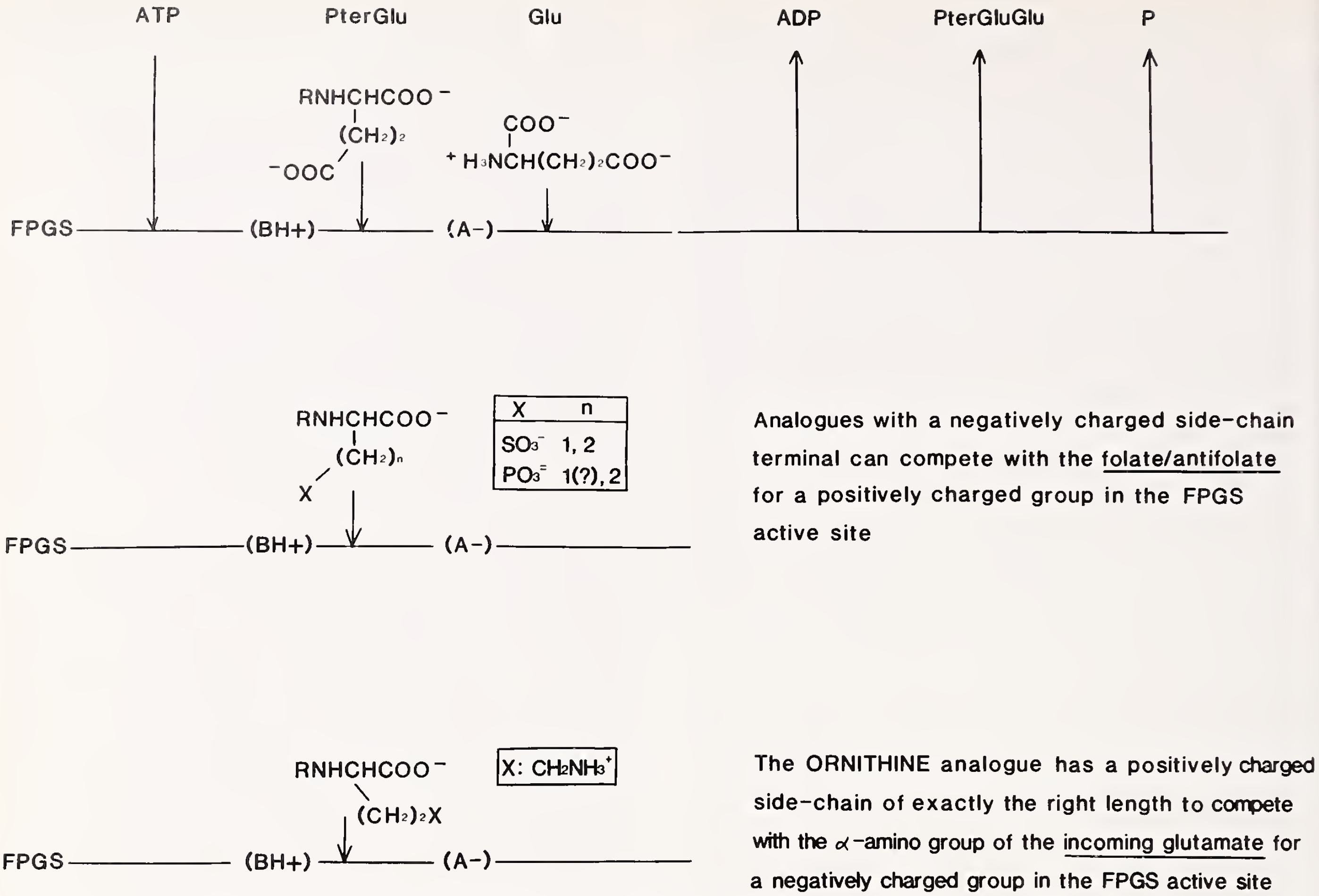
of a 100-fold excess of γ-fluoromethotrexate, MTX polyglutamylation is inhibited by only 20%. This γ-modified MTX analog is therefore a poor inhibitor, and while it has roughly the same activity as our γ-sulfonate analog, it is much less active than either the γ-phosphonate analogs or L-Orn analogs described here. The only FPGS inhibitor reported until now to have a K_i of the same order as APA-L-Orn is the corresponding analog of (6S)-tetrahydrofolate, with a K_i of 0.2 μM against enzyme purified from hog liver (*17*).

Several aspects of the data in table 2 merit discussion. One of these relates to the question of side-chain stereochemistry, another relates to the number of CH_2 groups, and the last relates to the mode of interaction of these compounds with FPGS. Each of these points will be considered briefly below.

With regard to the stereochemistry of the α-carbon, we observed earlier (*32*) that mAPA-L-HCysA and APA-L-HCysA had K_i of 198 ± 31 μM and 59 ± 28 μM, respectively. From these results and the data in table 2 it would appear that the L-enantiomers and D,L-racemates possess similar FPGS affinity in each series. A possible explanation of these unusual results is that racemization occurred during the synthesis of the L-enantiomers. While the complete absence of any racemization was not established, we feel that the L-configuration was largely preserved in the products because there was measurable optical activity and because the synthesis was intentionally conducted under mild, non-racemizing conditions. A second explanation of the results could be that the L- and D-enantiomers in fact have comparable affinities for the enzyme. This may seem surprising when one considers that the activity of D-MTX as a substrate for FPGS is only 1.9% of that of L-MTX (*29*). It may be noted, on the other hand, that D-MTX does have some activity as a competitive inhibitor even though it is a very poor substrate, and that this inhibitory activity is of the same order as that of MTX α-*tert*-butyl ester (blocked α-carboxyl) and 4-*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)aminobutyric acid (deleted α-carboxyl) (*29,30*). It is thus conceivable that compounds that are inhibitors interact with FPGS differently than compounds that are substrates, and that when compounds that are inhibitors bind to the enzyme the stereochemistry and perhaps even the nature of the substitutent on the α-carbon are less important than they are for binding of a substrate. In other words, an absolute requirement for the L-configuration at the α-carbon of a folate-antifolate may exist for substrate activity but not for enzyme binding per se. We are currently examining this question with other side-chain-altered analogs.

The second aspect of the FPGS inhibition data which is of interest relates to the length of the amino acid side chain. We reported previously that the HCysA and cysteic acid analogs of MTX had comparable K_i and that the same was true for the HCysA and cysteic acid analogs of AMT (*32*). This was surprising in view of the finding that APA-L-Asp, the L-aspartic acid analog of AMT, had almost no substrate activity in the FPGS assay (*29*). However, it was also found in the same study that the L-aspartic acid analog could decrease folate polyglutamylation by 50% when the substrate and inhibitor were present in equimolar amounts. It therefore appears that when an acidic terminal group is present in the FPGS inhibitor, there is some room for variation in the number of CH_2 units separating this group from the rest of the molecule. It is interesting to note, on the other hand, that this seems not to be true for inhibitors with a basic group at the terminal position; substitution of L-2,4-diaminobutyric acid, which contains one less CH_2, for L-Orn results in a >3000-fold decrease in anti-FPGS activity (*37*). This suggests that, in contrast to the γ-sulfonate and γ-phosphonate analogs, the side chain of mAPA-L-Orn and APA-L-Orn may project into a region of the FPGS active site which is sterically very crowded and has a low capacity for conformational change of the "induced fit" type in response to the molecular structure of the bound drug.

The fact that MTX and AMT analogs with either a basic or acidic substituent in the distal region of the side chain can bind strongly to FPGS raises questions as to how these compounds interact with the enzyme and why the L-Orn analogs are actually better inhibitors than the γ-sulfonates or γ-phosphonates. We have developed in this regard a working hypothesis which can be schematically formulated as in figure 4. The upper portion of the scheme, based on a prevailing view of the mechanism of FPGS-catalyzed polyglutamylation (*38*), depicts the sequence of steps leading to the γ-glutamylation of a pteridine substrate. After initial binding of an ATP molecule to a nucleotide-binding region, the pteridine binds to the enzyme in an orientation that allows the negatively charged γ-carboxyl to interact with a positively charged group (BH^+) (probably a protonated lysine or arginine side chain) in the active site. A phosphate group may be transferred to the γ-carboxyl at this time to form a reactive γ-acyl phosphate ester. It is unknown whether this occurs via direct transfer from ATP or whether there is an intermediate step involving phosphorylation of an enzyme amino acid residue. The next species that binds to the FPGS is Glu, and there is presumably a negatively charged group (A^-) in the active site with which the α-amino group of the incoming Glu can interact. It seems likely that BH^+ and A^- are close to each other since the α-amino group of Glu has to approach the γ-acyl phosphate moiety to attach to the pteridine. The BH^+ and A^- are probably also at about the same distance from whatever amino acid residues comprise the binding site for the pteryl moiety. Once the new amide bond to the second glutamate is formed and ADP departs from the enzyme, the pteridine followed by inorganic phosphate are released. In the middle and lower portions of figure 4 are shown inhibitors with a negatively charged side-chain terminus, as in the HCysA and APBA analogs, and inhibitors with a positively charged side-chain terminus, as in the Orn analogs. We are suggesting that the pteryl and α-carboxyl moieties of these 2 types of inhibitors probably bind similarly to the active site, but that the side chain can be projected in 1 direction (toward BH^+) or another (toward A^-) depending on the sign of the charge on X. Only an Orn side chain seems to be of the correct length to accommodate the interaction of not only the pteryl and α-carboxyl groups with their respective binding sites but also of the positively charged X group with A^-. A corollary of this model is that MTX and AMT analogs with side chains containing both acidic and basic groups at the correct distance from each other might be better inhibitors than analogs containing acidic or basic groups only. Work on compounds of this type is being undertaken to test this model.



Analogues with a negatively charged side-chain terminal can compete with the folate/antifolate for a positively charged group in the FPGS active site

The ORNITHINE analogue has a positively charged side-chain of exactly the right length to compete with the α-amino group of the incoming glutamate for a negatively charged group in the FPGS active site

FIGURE 4.—Possible mode of interaction of side-chain altered folate-antifolates with FPGS. Pte = pteryl.

CELL GROWTH INHIBITION

The ability of the D,L-APBA and L-Orn analogs of MTX and AMT to inhibit the growth of mammalian cells in culture was assayed in comparison with that of the parent drugs. The concentrations of each compound that inhibited cell growth by 50% after 48 hours of continuous treatment (IC_{50}) are given in table 3. The cells used were L1210 murine leukemia and a mutant subline (L1210/R81) with an extremely high level of MTX and AMT resistance based partly on DHFR overproduction but predominantly on a defect in the pathway for MTX active transport (*24*).

The D,L-APBA analogs of MTX and AMT were both less active than the parent drugs; mAPA-D,L-APBA was less potent than MTX by a factor of 100 and APA-D,L-APBA was less potent than AMT by a factor of 30. The decreased potency of these γ-phosphonate analogs may reflect in part the fact that they cannot form polyglutamates and therefore, in contrast to MTX and AMT, are unlikely to inhibit enzymes such as thymidylate synthase and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, which are somewhat inhibited by the polyglutamates of MTX (*39,40*) and presumably AMT. It is also possible, however, that decreased activity reflects, in part, inefficient binding to the carrier protein responsible for the active transport of MTX and reduced folates.

APA-D,L-APBA was 3 times more potent as an inhibitor of L1210 cell growth than was mAPA-D,L-APBA. Since the amino acid side-chains in these compounds are identical, it is clear that the N^{10}-substituent itself must contribute something to activity irrespective of polyglutamylation. We and others have proposed that the high FPGS-substrate activity of AMT in comparison with MTX contributes to the greater toxicity of AMT (*29,41,42*). Our data with the γ-phosphonate analogs of MTX and AMT suggest that this by itself may not be the sole reason for high toxicity of AMT, and that AMT ought to be more active than MTX

TABLE 3.—Inhibition of cell growth in culture by APBA and Orn analogs of MTX and AMT

	IC_{50}, μM^a	
Compound	L1210 cells	L1210/R81 cells
MTX	0.002	220 (110,000)
mAPA-D,L-APBA	0.20	76 (380)
mAPA-L-Orn	1.3	86 (66)
AMT	0.002	84 (42,000)
APA-D,L-APBA	0.067	240 (3,600)
APA-L-Orn	1.3	32 (25)

[a] Cells were cultured as previously described (*25*) and were counted after 48 hr of continuous exposure. *Numbers in parentheses* are relative to the IC_{50} for the same compound against wild-type cells.

even in the absence of polyglutamylation, e.g., in cells with a polyglutamylation defect (*9,43*) or in cells treated with a polyglutamylation inhibitor.

Replacement of Glu by Orn had a highly adverse effect on growth inhibition, the IC_{50} of mAPA-L-Orn and APA-L-Orn being 650 times greater than those of MTX and AMT. The Orn analogs were also less potent than the γ-phosphonates. If a self-potentiating antifolate effect (*13*) were operative in L1210 cells, one would expect the converse to be true, provided that the cells took up the same amount of drug. Since the Orn analogs were less potent than the γ-phosphonates, it is reasonable to conclude that they are probably less efficiently taken up by the cells. The net charge on the side-chain of the Orn analogs is zero, while in the γ-phosphonates there are 3 negative charges at physiological pH. It may be possible to improve the uptake of the Orn analogs by converting them to prodrug derivatives with a net negative charge which can mimic the dianion character of MTX and AMT.

In contrast to their low potency relative to MTX in the assay of L1210 cell growth, the D,L-APBA and L-Orn analogs of MTX were more potent than MTX against L1210/R81 cells; while the L1210/R81 cells were $>10^5$-fold MTX-resistant, they were only 380-fold resistant to mAPA-D,L-APBA and 66-fold resistant to mAPA-L-Orn. Replacement of Glu by Orn therefore decreased resistance by more than 3 orders of magnitude. A similar decrease in resistance was observed for APA-D,L-APBA and APA-L-Orn in comparison with AMT. Once again it should be noted that the data in table 3 for the γ-phosphonate analogs are for D,L-racemates; it is quite possible that the L-enantiomers, when they are made, will have a 2-fold lower IC_{50} than the D,L-racemates. The most potent compound against L1210/R81 cells was APA-L-Orn, which was also the most FPGS inhibitor. We believe that the decreased resistance of these cells to the D,L-APBA and L-Orn analogs in comparison with the parent drugs may reflect the ability of the side chain-altered analogs not only to inhibit DHFR, but also block the polyglutamylation of exogenous reduced folates. Since the affinity of the analogs for FPGS is considerably lower than their affinity for DHFR (for which they probably have a K_i in the subnanomolar range), self-potentiation would be expected to play a lesser role in MTX-sensitive cells than in cells which are MTX-resistant by virtue of an increase in DHFR content. In DHFR-overproducing cells, where much higher concentrations of the antifolate have to be reached to saturate the DHFR, enough free (i.e., not DHFR-bound) drug may accumulate in the cytoplasm to exceed the K_i for FPGS and thereby produce a significant reduction of the extent of polyglutamylation of the tetrahydrofolate cofactors involved in de novo DNA nucleotide synthesis. While our studies on self-potentiating dual inhibitors of DHFR and FPGS are still at an early stage, the data reported here provide encouragement for additional effort in this area of antifolate drug design.

REFERENCES

(*1*) Jolivet J, Cowan JH, Curt GA, et al: The pharmacology and clinical use of methotrexate. N Engl J Med 209: 1094–1103, 1983.

(*2*) Montgomery JA, Piper JR: Design and synthesis of folate analogs as antimetabolites. *In* Folate Antagonists as Therapeutic Agents (Sirotnak FM, Burchall JJ, Ensminger WB, et al, eds). Orlando, FL: Academic Press, 1984, pp 219–260.

(*3*) Rosowsky A, Beardsley GP, Ensminger WD, et al: Methotrexate analogues. 11. Unambiguous chemical synthesis and in vitro biological evaluation of α- and γ-monoesters as potential prodrugs. J Med Chem 21: 380–386, 1978.

(*4*) Rosowsky A, Lazarus H, Yuan GC, et al: Effects of methotrexate esters and other lipophilic antifolates on methotrexate-resistant human leukemic lymphoblasts. Biochem Pharmacol 29:580–589, 1980.

(*5*) Rosowsky A, Forsch RA, Uren J, et al: Methotrexate analogues. 14. Synthesis of new γ-substituted derivatives as dihydrofolate reductase inhibitors and potential anticancer agents. J Med Chem 24:1450–1455, 1981.

(*6*) Rosowsky A, Abelson HT, Beardsley GP, et al: Pharmacologic studies on the dibutyl and γ-monobutyl esters of methotrexate in the Rhesus monkey. Cancer Chemother Pharmacol 10:55–61, 1982.

(*7*) Rosowsky A, Forsch RA, Yu CS, et al: Divergent influence of alkyl chain-length on the dihydrofolate reductase affinity and cytotoxicity of methotrexate monoesters. J Med Chem 27:605–609, 1984.

(*8*) Rosowsky A, Freisheim JH, Bader H, et al: Methotrexate analogues. 25. Chemical and biological studies on the γ-tert-butyl esters of methotrexate and aminopterin. J Med Chem 28:660–667, 1985.

(*9*) Frei E III, Rosowsky A, Wright JE, et al: Development of methotrexate resistance in a human squamous cell carcinoma of the head and neck in culture. Proc Natl Acad Sci USA 81:2873–2877, 1984.

(*10*) Rosowsky A, Wright JE, Cucchi CA, et al: Phenotypic heterogeneity in cultured human head and neck squamous cell carcinoma lines with low-level methotrexate resistance. Cancer Res 45:6205–6212, 1985.

(*11*) Rosowsky A, Forsch RA, Uren J, et al: Methotrexate analogues. 20. Replacement of glutamate by longer-chain amino diacids: effects on dihydrofolate reductase inhibition, cytotoxicity, and in vivo antitumor activity. J Med Chem 26:1719–1724, 1983.

(*12*) Browman GP, Spiegl P, Booker P, et al: Comparison of leucovorin protection from a variety of antifolates in human lymphoid cell lines. Cancer Chemother Pharmacol 15:111–114, 1985.

(*13*) Rosowsky A, Moran RG, Forsch R, et al: Methotrexate analogues. 17. The antitumor activity of 4-amino-4-deoxy-N^{10}-methylpteroyl-D,L-homocysteic and its dual inhibition of dihydrofolate reductase and folyl polyglutamate synthetase. Biochem Pharmacol 33:155–161, 1984.

(*14*) Rosowsky A, Forsch RA, Freisheim JH, et al: Methotrexate analogues. 19. Replacement of the glutamate side-chain in classical antifolates by L-homocysteic acid and L-cysteic acid: effect on enzyme inhibition and antitumor activity. J Med Chem 27:600–604, 1984.

(*15*) Forsch RA, Freisheim JH, Moran RG, et al: γ-Phosphonate analogues of methotrexate (MTX) and aminopterin (AMT) as inhibitors of dihydrofolate reductase (DHFR) and folyl polyglutamate synthetase (FPGS). Proc Am Assoc Cancer Res 26:22, 1985.

(*16*) Rosowsky A, Moran RG, Houghton JA: Potent mechanism-based competitive inhibitors of murine and human folylpolyglutamate synthetase (FPGS). Pharmacologist 27:152, 1985.

(*17*) Cichowicz D, Cook J, George S, et al: Hog-liver folylpolyglutamate synthetase: substrate specificity and regulation. *In* Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates (Goldman ID, ed). New York: Praeger, 1985, pp 7–13.

(*18*) Rosowsky A, Wright JE, Ginty C, et al: Methotrexate analogues. 15. A methotrexate analogue designed for active-site-directed irreversible inactivation of dihydrofolate reductase. J Med Chem 25:960–964, 1982.

(*19*) Piper JR, Montgomery JA: Preparation of 6-(bromomethyl)-2,4-pteridinediamine hydrobromide and its use in improved syntheses of methotrexate and related compounds. J Org Chem 42:208–211, 1977.

(*20*) Yamada S, Ikota N, Shioiri T: Diphenyl phosphoroazidate (DPPA) and diethyl phosphorocyanidate (DEPC). Two new reagents for solid-phase peptide synthesis and their application to the synthesis of porcine motilin. J Am Chem Soc 97:7174–7175, 1975.

(*21*) Chaykovsky M, Rosowsky A, Papathanasopoulos NP, et al: Methotrexate analogs. 3. Synthesis and biological properties of some side chain altered analogs. J Med Chem 17:1212–1216, 1974.

(*22*) Chaykovsky M, Brown BL, Modest EJ: Methotrexate analogs. 6. Replacement of glutamic acid by various amino acid esters and amines. J Med Chem 18:909–912, 1975.

(*23*) Piper JR, McCaleb GS, Montgomery JA, et al: Syntheses and evaluation as antifolates of MTX analogues derived from 2,ω-diaminoalkanoic acids. J Med Chem 28:1016–1025, 1985.

(*24*) McCormick JI, Susten SS, Freisheim JH: Characterization of the methotrexate transport defect in a resistant L1210 lymphoma cell line. Arch Biochem Biophys 212:311–318, 1981.

(*25*) Susten SS, Kempton RJ, Black AM, et al: A fluorescent analogue of methotrexate as a probe for folate antagonist molecular receptors. Biochem Pharmacol 33:1957–1962, 1984.

(*26*) Cramer SM, Schornagel JH, Kalghatgi KK, et al: Occurrence and significance of D-methotrexate as a contaminant of commercial methotrexate. Cancer Res 44:1843–1846, 1984.

(*27*) Cohen M, Bender RA, Donehower R, et al: Reversibility of high-affinity binding of methotrexate in L1210 murine leukemia cells. Cancer Res 38:2866–2870, 1978.

(*28*) Jackson RC, Hart LI, Harrap KR: Intrinsic resistance to methotrexate of cultured mammalian cells in relation to the inhibition kinetics of their dihydrofolate reductases. Cancer Res 36:1991–1997, 1976.

(*29*) Moran RG, Colman PD, Rosowsky A, et al: Structural features of 4-amino antifolates required for substrate activity with mammalian folylpolyglutamate synthetase. Mol Pharmacol 27:156–166, 1985.

(*30*) Moran RG, Colman PD, Forsch RA, et al: A mechanism for the addition of multiple moles of glutamate by folylpolyglutamate synthetase. J Med Chem 27:1263–1267, 1984.

(*31*) Moran RG, Colman PD: Mammalian folylpolyglutamate synthetase. Partial purification and characterization of the mouse liver enzyme. Biochemistry 23:4580–4589, 1984.

(*32*) Moran RG, Rosowsky A, Colman P, et al: Structural features of folate analogs that determine substrate or inhibitor activity for mammalian folylpolyglutamate synthetase. *In* Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates (Goldman ID, ed). New York: Praeger, 1985, pp 51–64.

(*33*) McGuire JJ, Hsieh P, Bertino JR, et al: Studies on the substrate specificity of mammalian folylpolyglutamate synthetase. *In* Chemistry and Biology of Pteridines: Pteridine and Folic Acid Derivatives (Blair JA, ed). Berlin: de Gruyter, 1983, pp 615–619.

(*34*) Tang KC, Coward JK: Synthesis of acyl phosphonate analogues of biologically important acyl phosphates: *N*-(2-amino-10-methylpteroyl)-5-amino-2-oxopentanephosphonic acid. J Org Chem 48:5001–5006, 1983.

(*35*) McGuire JJ, Coward JK: D,L-Threo-4-fluoroglutamic acid: a chain-terminating inhibitor of folylpolyglutamate synthesis. J Biol Chem 260:6747–6754, 1985.

(*36*) Galivan J, Inglese J, McGuire JJ, et al: γ-Fluoromethotrexate: synthesis and biological activity of a potent inhibitor of dihydrofolate reductase with greatly diminished ability to form poly-γ-glutamates. Proc Natl Acad Sci USA 82:2598–2602, 1985.

(*37*) Rosowsky A, Freisheim JH, Moran RG, et al: Methotrexate analogues. 26. Inhibition of dihydrofolate reductase and folylpolyglutamate synthetase activity and in vitro tumor cell growth by methotrexate and aminopterin analogues containing a basic amino acid side chain. J Med Chem 29:655–660, 1986.

(*38*) Cichowicz DJ, Foo SK, Shane B; Folylpoly-γ-glutamate synthesis by bacteria and mammalian cells. Mol Cell Biochem 39:209–228, 1981.

(*39*) Allegra CJ, Drake JC, Jolivet J, et al: Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. Proc Natl Acad Sci USA 82:4881–4885, 1985.

(*40*) Allegra CJ, Chabner BA, Drake JC, et al: Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. J Biol Chem 260:9720–9726, 1985.

(*41*) Matherly LH, Voss MK, Anderson LA, et al: Enhanced polyglutamylation of aminopterin relative to methotrexate in the Ehrlich ascietes tumor cell in vitro. Cancer Res 45:1073–1078, 1985.

(*42*) Samuels LL, Moccio DM, Sirotnak FM: Similar differential for total polyglutamylation and cytotoxicity among various folate analogues in human and murine tumor cells in vitro. Cancer Res 45:1488–1495, 1985.

(*43*) Cowan KH, Jolivet J: A methotrexate-resistant human breast cancer cell line with multiple defects, including diminished formation of methotrexate polyglutamates. J Biol Chem 259:10793–10800, 1984.

Antifolate Metabolism and Sites of Action: Implications for the Design of New Antifolates

Anthony L. Fitzhugh,[1,*] Bruce A. Chabner,[2] Pam W. Sholar,[2] Jacob Baram,[2] and Carmen J. Allegra[2]

ABSTRACT—Recently, folate-dependent enzymes in the de novo thymidine and purine biosynthetic pathways have come under scrutiny as potential sites for chemotherapeutic exploitation by antifolates. In this manuscript we report on the progress that has been made in designing inhibitors of these pathways. In addition, a molecular model is proposed for the design of new antifolates directed against thymidylate synthase (TS).—NCI Monogr 5:153-157, 1987.

Ideally the rational design of antifolates for cancer chemotherapeutic purposes should depend on an intimate understanding of the molecular mechanisms of the important folate-dependent, rate-limiting steps in the biosynthesis of DNA precursors. However, methotrexate (MTX), or 4-amino-N^{10}-methylfolic acid (fig. 1), the paragon of antifolates, was brought to clinical trial (*1*) when little was known about its site of action or folate metabolism. Indeed, phenomena such as active transport (*2*), polyglutamylation (*3*), or the interconversion of various one-carbon units in the intracellular folate pool (*4*) had not yet been clarified. In later biochemical studies (*5,6*) it was discovered that MTX acted as a potent inhibitor [inhibitory constant (K_i) in the 10^{-11} *M* range] of dihydrofolate reductase (DHFR), an enzyme responsible for recycling 7,8-dihydrofolate (FH_2) to its reduced, physiologically active, 6(R)-tetrahydro form. As a result of this observation, the cytotoxic effect of MTX has been ascribed to the depletion of the intracellular pool of reduced folates. The search for even more tightly binding inhibitors of DHFR has led to the synthesis and biological evaluation of literally hundreds of analogs structurally related to MTX (*7*). With rare exception (*8,9*), these efforts have largely been a disappointment. Thus, almost 40 years after its introduction as a cancer chemotherapeutic agent, MTX remains the most important antifolate in clinical use.

Since numerous attempts to design inhibitors of DHFR clinically superior to MTX have met with little success, the need to design antifolates directed against loci other than DHFR has emerged. Here, too, MTX has played a major role. This is exemplified in our recent examination of the effect of MTX on intracellular folate pools in MCF-7 breast cancer cells (*10*), where MTX did not cause quantitative depletion of the reduced folate pools as might be expected following blockade of DHFR. Examination by high-pressure liquid chromatography of the intracellular folate pools revealed less than 50% depletion of 2 key reduced-folate cofactors used in thymidine and purine biosynthesis, namely N^5,N^{10}-methylenetetrahydrofolate and N^{10}-formyltetrahydrofolate. There was, however, ample evidence for the establishment of a block at the level of DHFR in the form of a rapid increase in the intracellular pools of FH_2 and N^{10}-formyldihydrofolate, changes that coincided temporally with a profound diminution in purine and thymidine biosynthesis some 2 hours after MTX exposure. At later time points (6 to 12 hr after MTX exposure), it was possible to demonstrate formation of MTX polyglutamates in considerable excess over the level of its monoglutamate form. A follow-up study (*11,12*) involving the direct assay of MTX and FH_2 polyglutamates demonstrated that these derivatives are excellent inhibitors of TS and 5-amino-imidazole-4-carboxamide ribonucleotide (AICAR) transformylase, with K_i's ranging from 10^{-6} to $10^{-8}M$ when tested against their polyglutamate or monoglutamate folate enzyme-substrate complexes. Similar results have been reported for MTX polyglutamates with glycinamide ribonucleotide (GAR) transformylase (*13*). These findings therefore strongly suggest that direct inhibition of TS and GAR/AICAR transformylase, first by FH_2 (or N^{10}-formyldihydrofolate) polyglutamates and later by MTX polyglutamates, may well be the primary cause of MTX cytotoxicity inasmuch as significant depletion of the reduced folate pools was not observed during any time interval studied. While the scope of these experiments was limited to the MCF-7 breast cancer cell line, they nevertheless underscore the importance of inhibition of these enzymes to antifolate cytotoxicity. In the present paper, we will review recent efforts to develop antifolates of these more "distal" enzymes and propose a molecular model of the folate-TS interaction that may be used as a basis for the design of new antifolates of TS.

ABBREVIATIONS: AICAR = 5-amino-imidazole-4-carboxamide ribonucleotide; DDIP = 5,8-dideazaisopteroyl monoglutamate; DDTHF = 5,10-dideazatetrahydrofolate; DHFR = dihydrofolate reductase; FdUMP = 2′-deoxy-5-fluorouridine monophosphate; FH_2 = 7,8-dihydrofolate; FUra = 5-fluorouracil; GAR = glycinamide ribonucleotide; IC_{50} = inhibitor concentration needed to inhibit 50% of enzyme activity; K_D = dissociation constant; K_i = inhibitory constant; MTX = methotrexate; N^5,N^{10}-CH_2-FH_4 = N^5,N^{10}-methylenetetrahydrofolate; PABA = *p*-aminobenzoyl; PDDF = N^{10}-propargyl 5,8-dideazafolate; THHF = tetrahydrohomofolate; TS = thymidylate synthase.

---

[1] Medicinal Chemistry Section, CSAL, PRI-NCI-Frederick Cancer Research Facility, Frederick, MD.

[2] Clinical Pharmacology Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[*] *Reprint requests to:* Anthony L. Fitzhugh, M.D., Medicinal Chemistry Section, PRI-NCI-Frederick Cancer Research Facility, PO Box B, Frederick, MD 21701.

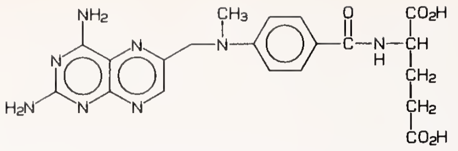


FIGURE 1.—Molecular structure of MTX.

ANTIFOLATES THAT INHIBIT PURINE BIOSYNTHETIC PATHWAYS

Three types of antifolates have recently been synthesized that act as inhibitors of purine biosynthesis. The first, the homofolates, represents a unique group of compounds with an additional methylene group between the pteridine and *p*-aminobenzoyl (PABA) rings of folic acid yielding a C^{10}, N^{11} structure. 7,8-Dihydrohomofolate is a substrate for DHFR (*14*), and its product tetrahydrohomofolate (THHF) may be naturally or synthetically converted to either the N^{11}-formyltetrahydrohomofolate or N^5,N^{11}-methenyltetrahydrohomofolate derivatives (fig. 2) (*15,16*). From studies in sarcoma 180 and HKSV 28 cells, it appears likely that an important site of action of these derivatives is inhibition of GAR transformylase. This is evidenced by the sharp decrease in the levels of formylglycinamide ribonucleotide that accompanies inhibition of cell growth in the presence of these derivatives. The inhibitory effect of the N^5,N^{11}-methenyl derivative, however, is not restricted to GAR transformylase because aminoimidazole carboxamide, given concurrently, does not prevent growth inhibition in HKSV 28 cells. To what degree the THHF series of compounds undergoes polyglutamylation in vitro is not known, but this process might well enhance their ability to inhibit additional sites along the purine biosynthetic pathway. A clinical trial of THHF has been attempted in a single patient with N^5-methyltetrahydrohomofolate, but was terminated for lack of pure compound (*17*). The second type is N^{10}-formyl-8-deazafolate, whose structure is shown in figure 3. Smith et al. (*18*) tested the monoglutamate derivative as part of an N^{10}-formyl panel of deazafolates and found that it acts as a good ($K_i \approx 10^{-6}$ *M*) inhibitor of purified GAR transformylase. Surprisingly, despite the well-accepted assumption that polyglutamylation lowers the K_i typically 10- to 1000-fold, no cell culture studies of this antifolate have been reported. The third and most promising type, 5,10-dideazatetrahydrofolate (DDTHF) (fig. 3), was synthesized by Taylor et al. (*19*). Initial studies (*20,21*) of the compound have revealed potent inhibition of cell growth both in L1210 cells [inhibitor concentration needed to inhibit 50% of enzyme activity (IC_{50}) in 10^{-8} *M* range] and in a number of solid tumors in mice. These effects are reversed by hypoxanthine or AICAR, suggesting that DDTHF also inhibits at the level of GAR transformylase. Although no clinical trials have been reported with 5,10-dideazatetrahydrofolate, the breadth of its activity against solid tumors holds tremendous therapeutic promise.

MOLECULAR MODEL FOR TS-FdUMP-CH_2-FH_4 INTERACTION AND ANTIFOLATES THAT INHIBIT THYMIDINE BIOSYNTHESIS

Studies at the molecular level of enzyme inhibitors often provide insights into the mechanism of the reaction. A particular type of inhibitor, termed transition-state inhibitor (*22*), may be designed to mimic the putative molecular configuration and electronic interactions of the enzyme-substrate complex as the substrate undergoes transformation to product. Since some transition-state inhibitor-enzyme complexes are stable, the manner in which the transition-state inhibitor binds to the enzyme can be analyzed in detail to yield clues as to the enzymatic mechanism and to suggest possible strategies for the design of potential inhibitors. One such transition-state inhibitor-enzyme complex that prevents the de novo biosynthesis of the important DNA precursor, thymidine monophosphate, is formed by TS, (+)N^5,N^{10}-methylene-tetrahydrofolate (N^5,N^{10}-CH_2FH_4) and 2′-deoxy-5-fluorouridine monophosphate (FdUMP), one of the active metabolites of FUra (*23*). Because of our interest in the development of antifolates, we have recently focused our attention on understanding

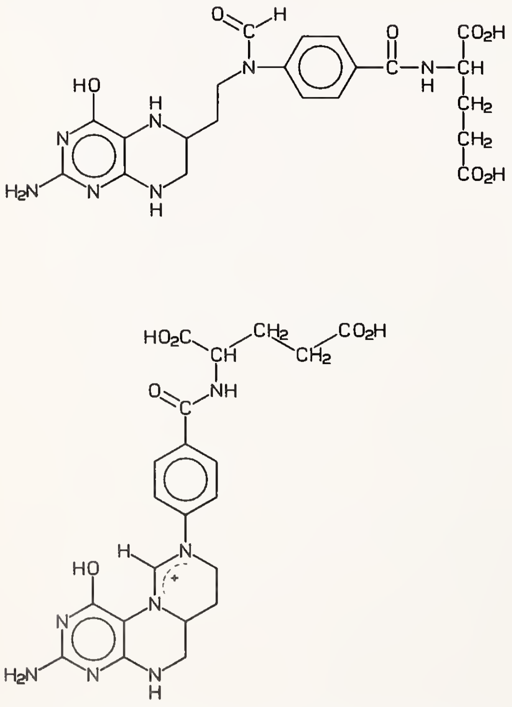


FIGURE 2.—Molecular structures of N^{11}-formyltetrahydrohomofolate (*top*) and N^5,N^{11}-methenyltetrahydrohomofolate (*bottom*).

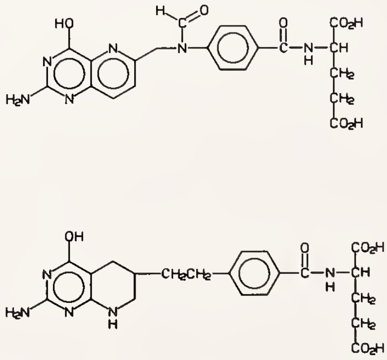


FIGURE 3.—Molecular structures of N^{10}-formyl-8-dideazafolate (*top*) and DDTHF (*bottom*).

the role of folate cofactor N^5,N^{10}-CH_2FH_4 during the inhibition of TS by FdUMP.

FdUMP alone is known to be a rather weak binder to TS, with a dissociation constant (K_D) of 10^{-5} *M*; however, in the presence of the cofactor, N^5,N^{10}-CH_2FH_4, a tight enzyme-FdUMP-cofactor ternary complex is formed (K_i of 10^{-12} *M*). Physical evidence for the transition-state structure proposed in figure 4 has been largely obtained from studies (*24,25*) of its peptide fragment (6–8 amino acid residue) formed following proteolysis of the native ternary complex from the bacterium *Lactobacillus casei*. However, when a UV spectrum of the native ternary complex was obtained, 2 unexpected absorption maxima at 322 and 375 nm were observed, both of which were found to disappear instantaneously upon denaturation (*26*). Also, in contrast to the irreversibly linked peptide fragment, the native ternary complex underwent a slow reversal with a half-life of 10 hours (*27*).

The structure in figure 4 does not account for the absorption maxima at 322 and 375 nm and/or the reversibility of the ternary complex when in the native state. Fitzhugh et al. (*28*) therefore undertook a resonance-Raman spectroscopic investigation of the native ternary complex using laser excitation at 337 and 356 nm. [This technique gives the vibrational spectrum of the chromophore responsible for the unknown UV band(s). By direct comparison with the vibrational spectrum of a known chromophore(s), one can typically obtain evidence for the chromophore giving rise to an unknown UV spectral pattern.] From analysis of the resonance-Raman spectra, it seems likely that the PABA portion of the cofactor is in fact the primary chromophore responsible for the anomalous UV band(s) at 322 and 375 nm observed in the native ternary complex. Significantly, the resonance-Raman spectrum of the 322 nm chromophore indicates that there is partial redistribution of the lone pair electron density at the N^{10} nitrogen of the PABA group of the cofactor into its benzoyl ring, i.e., the PABA group is serving as an "electron sink" for the lone pair electrons at N^{10}, giving it a quininoid-like structure (fig. 5).

On the basis of these observations, a revision of our current model is in order because there now appear to be factors from both the antimetabolite (FdUMP) and the cofactor (CH_2FH_4) that result in the stabilization of the native ternary complex. They are: 1) the inability of TS to abstract the fluorine atom as F^+, preventing the forward reaction from proceeding beyond the formation of the ternary complex; and 2) the perturbed electronic environment of the N^{10} nitrogen, which, when partially electropositive, slows restoration of the N^5,N^{10}-CH_2 bridge in the cofactor and thereby dissociation of the native ternary complex.

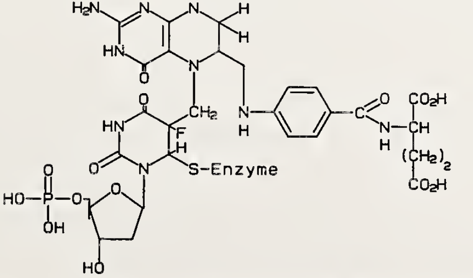


FIGURE 4.—Current model of the native 5,10-CH_2-FH_4/FdUMP/TS ternary complex.

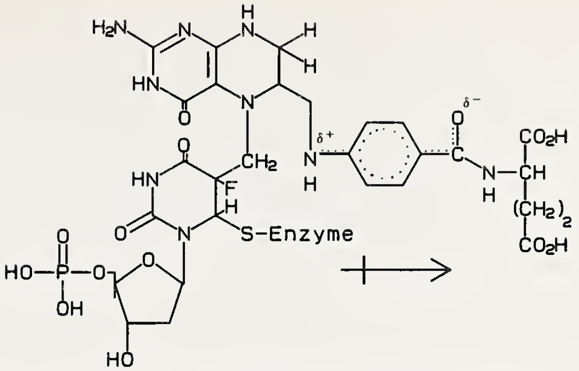


FIGURE 5.—Revised model of the native 5,10-CH_2-FH_4/FdUMP/TS ternary complex showing partial polarization in the PABA portion of the cofactor.

This discovery suggests that antifolates designed to promote and stabilize a partial positive charge on the N^{10}-nitrogen atom of the PABA group will enhance their transition-state interaction with TS and thus their inhibitory potency. Evidence supporting the aforementioned concept can be gleaned from a comparison of the inhibitory action of two existing antifolates that have a high degree of specificity towards TS. Their structures are shown in figure 6. The monoglutamate of N^{10}-propargyl-5,8-dideazafolate (PDDF) is a potent inhibitor of TS from L1210 cells (*29*) and *L. casei* (*30*), with K_i in the 10^{-9} *M* range. 5,8-Dideazaisopteroyl monoglutamate (DDIP), however, is a weak inhibitor (*31*) with a K_i of 7×10^{-6} *M*, a difference on the order of 600-fold. These results are readily explicable because the propargyl group of PDDF inductively promotes the development of a partial positive charge on the N^{10} nitrogen atom and therefore would more closely resemble the proposed transition-state interaction of the cofactor, whereas DDIP, absent an electron lone pair at the crucial 10-position, would be expected to give the weak inhibitory activity observed. Of particular note, the triglutamyl derivative of DDIP (*31*), has considerable TS inhibitory activity (K_i of 9×10^{-8} *M*), a finding consistent with the well-established preference of TS for polyglutamates (*32,33*).

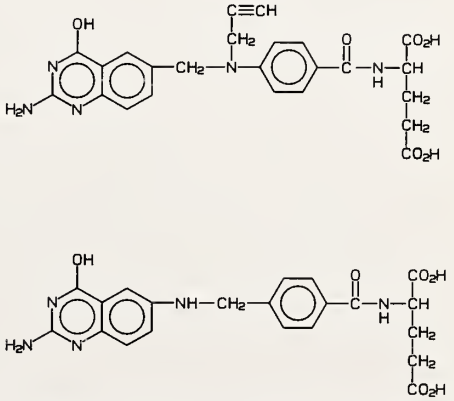


FIGURE 6.—Molecular structures of PDDF (*top*) and DDIP (*bottom*).

The selectivity of action of the more promising PDDF against TS has been indicated by the reversal of its cytotoxicity in culture by thymidine and its retention of activity against a MTX-resistant subline of L1210 cells that had amplified DHFR activity (*29*). An initial clinical trial of PDDF has produced complete or partial responses in 2 of 30 patients with ovarian cancer, 3 of 8 with breast cancer, and 1 response in lung cancer and mesothelioma (*34*).

SUMMARY AND COMMENTS

Largely on the basis of studies of the intracellular effect of MTX on reduced folate pools, the importance of inhibition of the thymidine and purine biosynthetic pathways to the development of cell cytotoxicity was discovered. In this manuscript we cited a number of recently synthesized antifolates in which site-specific inhibition has been achieved, excellent examples being PDDF and DDTHF. Unfortunately, however, none of the new antifolates have been adequately studied clinically or with respect to their intracellular mode(s) of action. Given the rich history of antifolate use and our increasing ability to define the electronic/molecular features needed in inhibitor design, the development of therapeutically effective agents should be realizable.

REFERENCES

(*1*) Burchenal JH, Karnofsky DA, Southam CM, et al: Clinical experience with the 4-amino derivatives of pteroylglutamic acid and 2,6-diaminopurine in the treatment of neoplastic disease. Am J Med 7:420, 1949.

(*2*) Dembo M, Sirotnak FM: Membrane transport in mammalian cells. *In* Folate Antagonists as Therapeutic Agents: Biochemistry, Molecular Actions and Synthetic Design. (Sirotnak FM, Burchall JJ, Ensminger WB, et al, eds), vol 1. New York: Academic Press, 1984, pp 173–217.

(*3*) McGuire JT, Bertino JR: Enzymatic synthesis and function of polyglutamates. Mol Cell Biochem 38:19–48, 1981.

(*4*) Kisliuk R: The biochemistry of folates. *In* Folate Antagonists as Therapeutic Agents: Biochemistry, Molecular Actions and Synthetic Design. (Sirotnak FM, Burchall JJ, Ensminger WB, et al, eds) vol 1. New York: Academic Press, 1984, pp 1–68.

(*5*) Nichol CA, Welch AD: On the mechanisms of action of aminopterin. Proc Soc Exp Biol Med 74:403–411, 1950.

(*6*) Osborn MJ, Freeman M, Huennekens FM: Inhibition of dihydrofolate reductase by aminopterin and amethopterin. Proc Soc Exp Biol Med 97:429–431, 1958.

(*7*) Sirotnak FM, DeGraw JI: Selective antitumor action of folate analogs. *In* Folate Antagonists as Therapeutic Agents: Pharmacology, Experimental and Clinical Therapeutics. (Sirotnak FM, Burchall JJ, Ensminger WB, et al, eds) vol 2. New York: Academic Press, 1984, pp 43–95.

(*8*) Burchall JJ, Hitchings GH: Inhibitor binding analysis of dihydrofolate reductases from various species. Mol Pharmacol 1:126–136, 1965.

(*9*) Bertino JR, Sawicki WL, Moronson BA, et al: 2,4-Diamino-5-methyl-6[3,4,5-trimethoxyanilino)methyl]-quinazoline (TMQ), a potent non-classical folate antagonist inhibitor. Biochem Pharmacol 28:1983–1987, 1979.

(*10*) Allegra CJ, Fine RL, Drake JC, et al: The effect of methotrexate on intracellular folate pools in human MCF-7 breast cancer cells: Evidence for direct inhibition of purine synthesis. J Biol Chem 261:6478–6485, 1986.

(*11*) Allegra CJ, Chabner BA, Drake JC, et al: Enhanced inhibition of thymidylate synthetase by methotrexate polyglutamates. J Biol Chem 260:9720–9726, 1985.

(*12*) Allegra CJ, Drake JC, Jolivet J, et al: Inhibition of AICAR transformylase by methotrexate and dihydrofolic acid polyglutamates. Proc Natl Acad Sci USA 82: 4881–4885, 1985.

(*13*) Allegra CJ, Drake JC, Jolivet J, et al: Inhibition of the folate-dependent enzymes of the de novo purine synthesis and folate interconverting enzymes by methotrexate polyglutamates (MTX-PGs). Proc Am Assoc Cancer Res 25:6, 1984.

(*14*) Divekar AY, Hakala MT: Inhibition of the biosynthesis of 5′-phosphoribosyl-*N*-formylglycinamide in sarcoma 180 cells by homofolate. Mol Pharmacol 11:319–325, 1975.

(*15*) Caperelli CA: Synthesis of 5,11-methenyltetrahydrohomofolate and its antifolate activity in vitro. J Med Chem 24:1086–1088, 1981.

(*16*) Slieker LJ, Benkovic SJ: Inhibition of HKSV28 cell growth by 5,11-methenyltetrahydrohomofolate. Mol Pharmacol 25:294–302, 1984.

(*17*) Cohen GI, Parker LM, Rosowsky A, et al: 5-Methenyltetrahydrohomofolate (MTHHF): Phase I trial and pharmacology in man. Proc ASCO 1:14, 1982.

(*18*) Smith GK, Mueller WT, Benkovic PA, et al: On the cofactor specificity of glycinamide ribonucleotide and 5-amino-imidazole-4-carboxamide ribonucleotide transformylase from chicken liver. Biochemistry 20:1241–1245, 1981.

(*19*) Taylor EC, Harrington PJ, Fletcher SR, et al: Synthesis of the antileukemic agents 5,10-dideaza-aminopterin and 5,10-dideaza-5,6,7,8-tetrahydroaminopterin. J Med Chem 28:914–922, 1985.

(*20*) Moran RG, Taylor EC, Beardsley GP: 5,10-Dideaza-5,6,7,8-tetrahydrofolic acid (DAHF), a potent antifolate inhibitory to de novo purine synthesis. Proc Am Assoc Cancer Res 26:231, 1985.

(*21*) Beardsley GP, Taylor EC, Harrington PJ, et al: A new class of antifolates. 5,10-Dideazatetrahydrofolic acid (DDATHF), an inhibitor of GAR transformylase with broad in vivo activity. Proc Am Assoc Cancer Res 27:259, 1986.

(*22*) Coward JK: Transition-state theory and reaction mechanism in drug action and design. *In* Transition States of Biochemical Processes (Gandour RD, Schowen RL, eds). New York: Plenum Press, 1978, pp 579–591.

(*23*) Heidelberger C: Fluorinated pyrimidines and their nucleosides. *In* Antineoplastic and Immunosuppressive Agents (Sartorelli AC, Johns DG, eds). New York: Springer-Verlag, 1975, pp 193–231.

(*24*) Santi DV, Pogolotti AL, James TL, et al: Thymidylate synthetase: Interaction with fluoro and 5-trifluoromethyl-2-deoxyuridylic acid. *In* Biochemistry Involving Carbon-Fluorine Bonds (Filler R, ed). Washington, DC: American Chemical Society, 1976, pp 57–76.

(*25*) Somer H, Santi DV: Purification and amino acid analysis of an active site peptide from thymidylate synthetase containing covalently bound 5-fluoro-2′-deoxyuridylate and methylenetetrahydrofolate. Biochem Biophys Res Commun 57:689–695, 1974.

(*26*) Donato H, Aull JL, Lyon JA, et al: Formation of ternary complexes of thymidylate synthetase as followed by absorbance, fluorescence, and circular dichroic spectra and gel electrophoresis. J Biol Chem 251:1303–1310, 1976.

(*27*) Santi DV, Pogolotti AL, Newman EM, et al: Aspects of the biochemistry and biochemical pharmacology of 5-fluorinated pyrimidines. *In* Biochemical Aspects of Fluorine Chemistry (Filler R, Kobayashi Y, eds). New York: Elsevier Biomedical, 1982, pp 123–142.

(*28*) Fitzhugh AL, Fodor S, Kaufman S, et al: Resonance-Raman spectroscopic evidence for alternative structures in the native ternary complex formed with thymidylate synthase. J Am Chem Soc 108:7422–7424, 1986.

(*29*) Jones TR, Calvert AH, Jackman AL, et al: A potent antitumor quinazoline inhibitor of thymidylate synthetase: synthesis, biological properties and therapeutic results in mice. Eur J Cancer 17:11–19, 1981.

(*30*) Kisliuk RL, Gaumont Y, Kumar P, et al: The effect of polyglutamylation on the inhibitory activity of folate analogs. *In* Second Workshop of Folyl and Antifolyl Polyglutamates (Goldman ID, ed). New York: Praeger, 1985, pp 319–328.

(*31*) Fernandes DJ, Bertino JR, Hynes JB: Biochemical and antitumor effects of 5,8-dideazaisopteroylglutamate, a unique quinazoline inhibitor of thymidylate synthase. Cancer Res 43:1117–1123, 1983.

(*32*) Dolnick BJ, Cheng YC: Human thymidylate synthetase. II. Derivatives of pteroylmono- and polyglutamates as substrates and inhibitors. J Biol Chem 253:3563–3567, 1978.

(*33*) Kisliuk RL, Gaumont Y, Baugh CM: Polyglutamyl derivatives of folate as substrates and inhibitors of thymidylate synthetase. J Biol Chem 249:4100–4103, 1974.

(*34*) Jackman AL, Taylor GA, Calvert AH, et al: Modulation of antimetabolite effects: effects of thymidine on the efficacy of the quinazoline-based thymidylate synthetase inhibitor CB3717. Biochem Pharmacol 33:3269–3275, 1984.

Biochemical Rationale for the Synergism of 5-Fluorouracil and Folinic Acid[1]

Richard G. Moran[2,3,*] and Khandan Keyomarsi[4]

ABSTRACT—The fluoropyrimidines, FUra and 5-fluoro-2′-deoxyuridine (FUdR), have been found to be more growth inhibitory and cytotoxic to both mouse and human tumor cells when grown in cell culture medium containing folinic acid. The increment in the activity of these drugs observed in folinate-containing medium was similar for a mouse leukemia cell line and for 4 human leukemia cell lines. This suggests that the mechanism of action of the fluoropyrimidines against these mouse and human cell lines is similar. The most probable mechanism of the interaction between folinic acid and the fluoropyrimidines is stabilization of thymidylate synthase (TS) in inactive complexes with 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) and folate cofactor. Such trapping of enzyme in inactive form would negate the effects of the accumulation of the reaction substrate 2′-deoxyuridine-5′-monophosphate. It is suggested that the combination of FUra with folinic acid and, in addition, an inhibitor of ribonucleotide reductase such as hydroxyurea may be more effective than FUra and folinic acid alone.—NCI Monogr 5:159-163, 1987.

FUra is a major chemotherapeutic agent used for the treatment of many adult carcinomas (*1,2*). The 2′-deoxyribonucleoside of FUra, FUdR, is a compound of considerable theoretical interest as a potent inhibitor of TS and has been used for the treatment of hepatic metastases from colorectal carcinoma (*3*). These fluoropyrimidines have a rather broad spectrum of activity against transplanted rodent tumors that are usually found to be predictive of the sensitivity of human disease (*4-6*). Yet, their activity against even colorectal carcinoma, the disease for which they are most often used, is limited to a fraction (about 20%) of individuals, and sufficient activity to result in the complete remission of an individual tumor is uncommon. The fluoropyrimidines have not proven to be useful for the treatment of human leukemias (*7,8*), yet have good activity against murine models of acute leukemia (*5,6*). The reason(s) for this disparity in the response of transplanted rodent tumors and (autochronous) human tumors to these drugs has not been satisfactorily explained.

Houghton et al. (*9*) made an observation that may prove germinal to an understanding of the limited sensitivity of human tumors to the fluoropyrimidines. During this course of studies on the response of several xenografts of human colorectal carcinoma to FUra, they found that FdUMP had limited effect on its target enzyme TS when added to cytosol fractions of 4 of these tumors and that these tumors did not respond to FUra. Addition of FdUMP to cytosol preparations of 2 human tumor lines that were drug sensitive resulted in complete inhibition of TS. However, when FdUMP and exogenous reduced folate cofactor were added to cytosols prepared from drug-refractory tumors, TS was inhibited completely. Such studies have led to the concepts: a) that the responsiveness of human tumors to FUra is limited by the availability of intracellular folate cofactor pools, and b) that expansion of the tumor content of 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4PteGlu) by supply of exogenous reduced folates would improve the efficacy of FUra against human tumors.

Ullman et al. (*10*) had previously shown that the concentrations of FUdR that produced inhibition of the growth of cultured mouse L1210 cells were lower in medium supplemented with folinic acid than in unsupplemented medium. This observation was confirmed and extended by Hakala and her colleagues who demonstrated (*11*) that the growth inhibition of both S180 mouse mammary carcinoma cells and Hep-2 human carcinoma cells caused by FUra was enhanced in the presence of folinic acid. Hakala and her colleagues (*11*) have suggested that, when folinic acid was added to the growth medium of human tumor cells, the site of inhibition of FUra changed from alteration of the function of RNA to inhibition of TS. These conclusions were supported by the observation that thymidine, which can clearly bypass inhibition of TS under some circumstances (*12*), did not relieve the growth inhibitory effects of FUra to Hep-2 cells in basal medium but did so to some degree in medium supplemented with folinic acid (*11*).

EFFECTS OF FUra AND FOLINIC ACID AGAINST MOUSE AND HUMAN LEUKEMIA CELL LINES

The growth-inhibitory and cytotoxic effects of FUra and of FUdR have been studied in cultures of a mouse leukemia cell line, the L1210 cell, and in four human leukemia cell lines (*13*). Two of these human cell lines were of the T-cell lineage (the CCRF-CEM and MOLT-4 cells) and two were B-cells (the CCRF-SB and B-8392 cells). The results of the

---

ABBREVIATIONS: 5,10-CH_2-H_4PteGlu = 5,10-methylenetetrahydrofolate; FdUMP = 5-fluoro-2′-deoxyuridine-5′-monophosphate; FUdR = 5-fluoro-2′-deoxyuridine; GZ = guanazole; H_4dUMP = tetrahydrodeoxyuridylate; H_4dUrd = tetrahydrodeoxyuridine; HU = hydroxyurea; TS = thymidylate synthase.

[1] Supported in part by Grant CA-36054 from the National Cancer Institute, Department of Health and Human Services.

[2] Division of Hematology-Oncology, Childrens Hospital of Los Angeles, and Departments of Pediatrics and Biochemistry, School of Medicine, University of Southern California, Los Angeles, CA.

[3] Scholar of the Leukemia Society of America.

[4] Department of Biochemistry, School of Medicine, University of Southern California, Los Angeles, CA.

* *Reprint requests to:* Richard G. Moran, Ph.D., Division of Hematology-Oncology, Childrens Hospital of Los Angeles, 4650 Sunset Boulevard, Los Angeles, CA 90054-0700.

growth-inhibitory studies are summarized in table 1. The growth-inhibitory potency of both compounds was increased by inclusion of folinic acid in the medium for all cell lines studied. Hence, it seems that this synergism is a general phenomenon and that folinic acid increases the effects of both FUra and FUdR against human leukemias. There seemed to be a trend in these data that the effects of FUdR were enhanced to a greater extent by folinic acid than were the effects of FUra. In addition, the degree of enhancement of the potency of FUra was similar in mouse and human cells. This effect is also seen in the data previously published by Hakala's group (*11*). It seems difficult to reconcile this fact with the concept (*11*) that the mechanism of FUra activity in mouse cells is inhibition of TS, but in human cells is RNA function. The enhancement of fluoropyrimidine effects by folinic acid can be explained in terms of the mechanism of inhibition of TS; there is no obvious mechanism whereby folinic acid could stimulate inhibition of RNA function.

PROBABLE MECHANISMS OF INTERACTION BETWEEN THE EFFECTS OF FOLINIC ACID AND THE FLUOROPYRIMIDINES

Folinic acid can be used as a sole source of folate for mammalian cells (*11,14*) and, therefore, can be converted to all of the cofactor forms required by the folate-dependent biosynthetic enzymes, including TS. The synthesis of thymidylate during the TS reaction seems to be a 2-step process (*15*): the first reaction involves the formation of a covalent complex between TS and its 2 substrates, dUMP and 5,10-CH_2-H_4PteGlu. The second step involves the transfer of a hydride ion from the 6-position of the cofactor with the formation of the 5-methyl group of thymidylate and release of free enzyme from the covalently bound transition state. There is substantial evidence that the inactivation of TS by FdUMP is due to the formation of a ternary complex between TS, FdUMP, and 5,10-CH_2-H_4PteGlu that is related in structure to that of the transition state (fig. 1). Danenberg and his colleagues (*16,17*) have furnished convincing evidence that the formation of both transition state and ternary complex proceed by an ordered sequential addition mechanism with nucleotide monophosphate combining with TS first (fig. 2). Although both the FdUMP–inhibition ternary complex and the transition state of the TS reaction represent covalently bound species, both complexes would dissociate to uninhibited free enzyme with a finite probability. For instance, the exchange of unlabeled FdUMP with [^{3}H]FdUMP in a preformed ternary complex can be easily demonstrated and, in fact, proceeds at a surprisingly rapid rate (*16,17*). Lockshin and Danenberg (*17*) demonstrated that when enzyme purified from CCRF-CEM cells was incubated with excess FdUMP and 5,10-CH_2-H_4PteGlu and the resultant covalently bound ternary complex was incubated in buffer in the absence of added cofactor, [^{3}H]FdUMP was released with a half-time of about 50 minutes. However. [^{3}H]FdUMP was lost increasingly slowly in the presence of increasing concentrations of 5,10-CH_2-H_4PteGlu. This effect was predictable because of the ordered sequential mechanism of formation of ternary complex. Thus, as ternary complex dissociates to the TS–FdUMP binary complex in these experiments, the probability or reformation of ternary complex becomes increasingly higher as 5,10-CH_2-H_4PteGlu concentration is raised (fig. 2). Presumably, this stabilization of the ternary complex also occurs in vivo after exposure of tumor cells to folinic acid and fluoropyrimidines. Lockshin and Danenberg (*17*) have presented evidence that FdUMP is probably not a tightly binding inhibitor of TS under physiological conditions except in the presence of high concentrations of folate cofactors.

Accumulation of dUMP

Inhibition of TS in vivo initiates the accumulation of the substrate of the TS reaction, dUMP (*18–21*). Presumably, this effect is due to the fact that the 2 enzymes that are rate limiting for dUMP synthesis (ribonucleotide reductase and deoxycytidylate deaminase) are controlled not by the concentration of dUMP itself, but rather by deoxyribonucleoside triphosphates, mainly dTTP (*22*). Hence, the control mechanisms that govern the rate of synthesis of dUMP probably allow decreased feedback inhibition when TS is inhibited and, thus, stimulate accumulation of dUMP. Because the binary association constants of either dUMP or FdUMP with TS are equivalent, a sufficiently expanded pool of dUMP would be expected to reverse the initial inhibition of TS by FdUMP. Thus, whether a cell population is killed by FUra or FUdR depends on the time course of an intrinsically transient effect—the temporary inhibition of TS prior to the accumulation of dUMP and recovery of enzyme activity. Tumor cell kill due to inhibition of TS following fluoropyrimidine exposure would reflect the interplay of several factors: 1) The extent of initial inhibition of enzyme, which reflects the amount of FdUMP made, and the available pools of 5,10-CH_2-H_4PteGlu which are present intracellularly as polyglutamates (*23,24*); 2) The stability of the ternary complex, once formed. which reflects primarily the availability of 5,10-CH_2-H_4PteGlu; 3) The rate of accumulation of dUMP, which will depend on large part on the presence of alternate pathways of loss of dUMP, such as endogenous phosphatases; and 4) The rate of irreversible damage to the cell population under thymineless conditions.

TABLE 1.—Augmentation of the growth-inhibitory effects of the fluoropyrimidines in the presence of folinic acid

Cell line	Ratio IC-50^{a,b}(+folinic acid)/ IC-50^b(−folinic acid)	
	FUra	FUdR
Mouse leukemic cells		
L1210	2.1	2.8
Human leukemic cells		
CCRF-CEM	1.7	3.3
MOLT-4	1.6	4.3
CCRF-SB	1.5	2.4
B-8392	2.0	4.6

[a] IC-50=The concentration of either FUra or FUdR that inhibited cell growth by 50% after 72 hours (L1210 cells) or 144 hours (human cell lines).

[b] Measured either in RPMI-1640 with dialyzed fetal calf serum or in the same medium supplemented with $10^{-5}M$ folinic acid. Data were drawn from Keyomarsi and Moran (*13*).

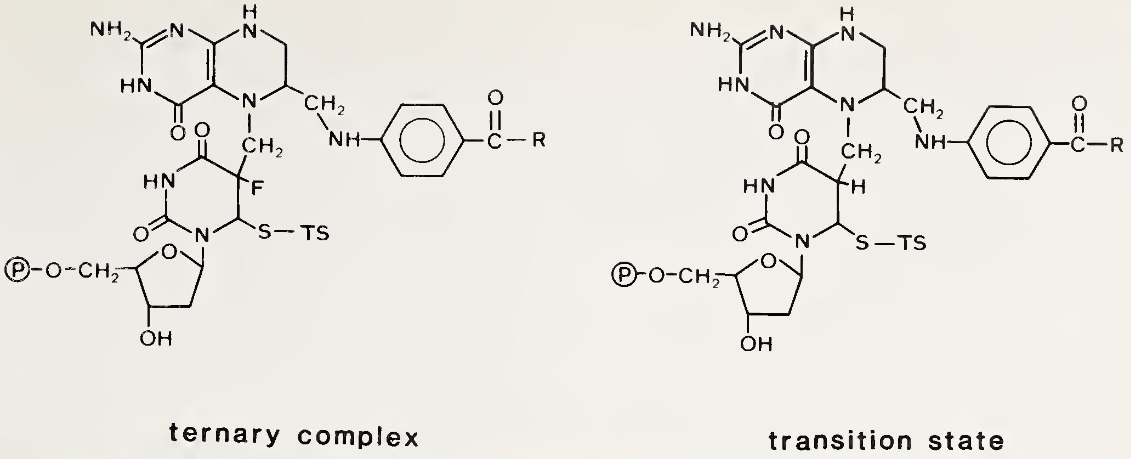


FIGURE 1.—Structures of the transition state of the TS reaction and of the ternary complex of TS, FdUMP, and 5,10-CH_2-H_4PteGlu.

Therapeutic Potential of Inhibition of dUMP Synthesis

A reasonable therapeutic approach to enhanced efficacy of the fluoropyrimidines is the pharmacological prevention of the accumulation of dUMP. Inhibition of early steps of pyrimidine biosynthesis with *N*-phosphonoacetyl-L-aspartate or pyrazofurin has been combined with fluoropyrimidines to decrease intracellular uridine nucleotide pools that might compete with FUra metabolites for incorporation into RNA and deoxyribonucleotide formation (*25–27*). An alternative approach is the coadministration of specific inhibitors of the 2 routes of dUMP synthesis, namely ribonucleotide reductase and deoxycytidylate deaminase. Several inhibitors of ribonucleotide reductase are known and 2 of these, hydroxyurea (HU) and guanazole (GZ), have been used clinically. Only 1 potent inhibitor of deoxycytidylate deaminase has been described, tetrahydrodeoxyuridylate (H_4dUMP) (*28*). As a nucleotide, H_4dUMP would not be expected to traverse the cell membrane. However, the corresponding deoxyribonucleoside, H_4dUrd, has been reported to be converted to H_4dUMP by cytosol fractions of a number of mouse tumors and of one human leukemia cell line, the CCRF-CEM (*29*). We have previously used FUra and FUdR in combination with HU, GZ, and H_4dUrd for

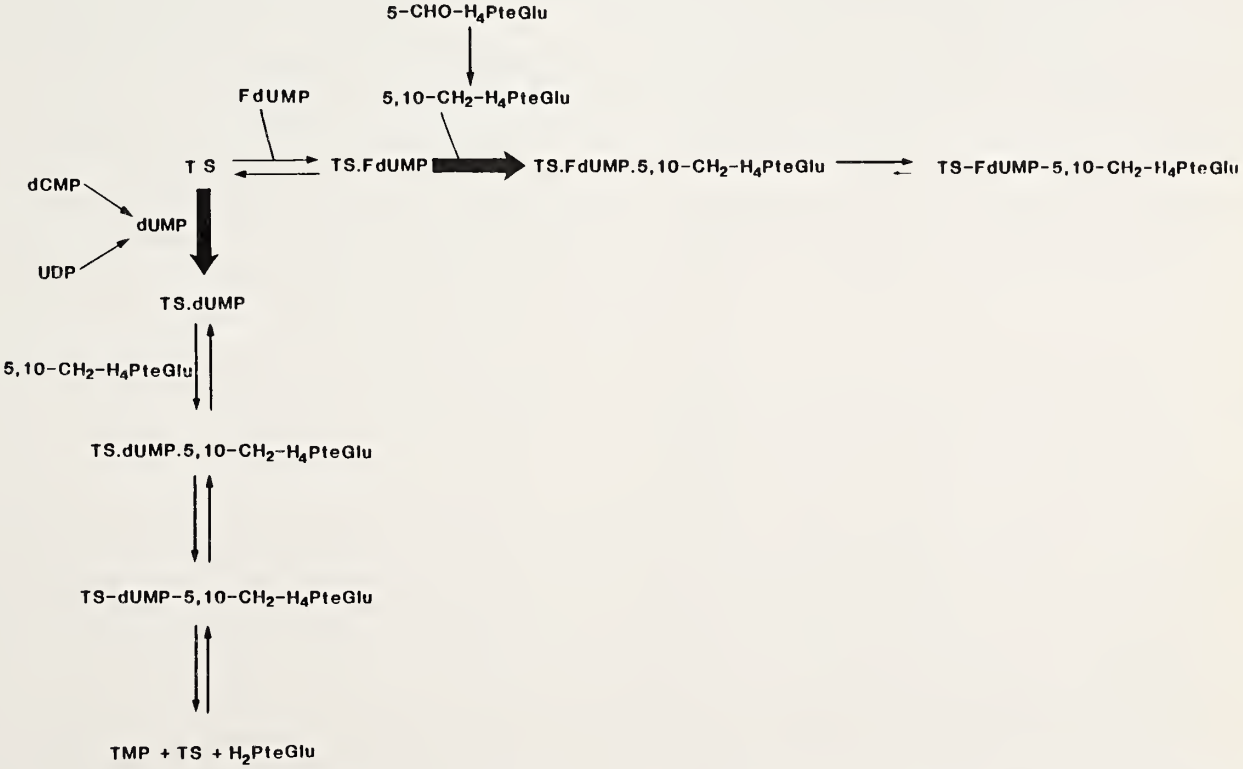

5-CHO-H4PteGlu
FdUMP
5,10-CH2-H4PteGlu
T S
TS.FdUMP
TS.FdUMP.5,10-CH2-H4PteGlu
TS-FdUMP-5,10-CH2-H4PteGlu
dCMP
dUMP
UDP
TS.dUMP
5,10-CH2-H4PteGlu
TS.dUMP.5,10-CH2-H4PteGlu
TS-dUMP-5,10-CH2-H4PteGlu
TMP + TS + H2PteGlu

FIGURE 2.—Sequence of addition of dUMP, FdUMP, and 5,10-CH_2-H_4PteGlu to TS. The addition of 5,10-CH_2-H_4PteGlu to the TS. FdUMP binary complex and of dUMP to free TS are shown by large 1-direction *arrows* to indicate that, in the presence of expanded pools of these metabolites, these steps are probably fast and essentially irreversible. Expanded cofactor pool prevents dissociation to free TS.

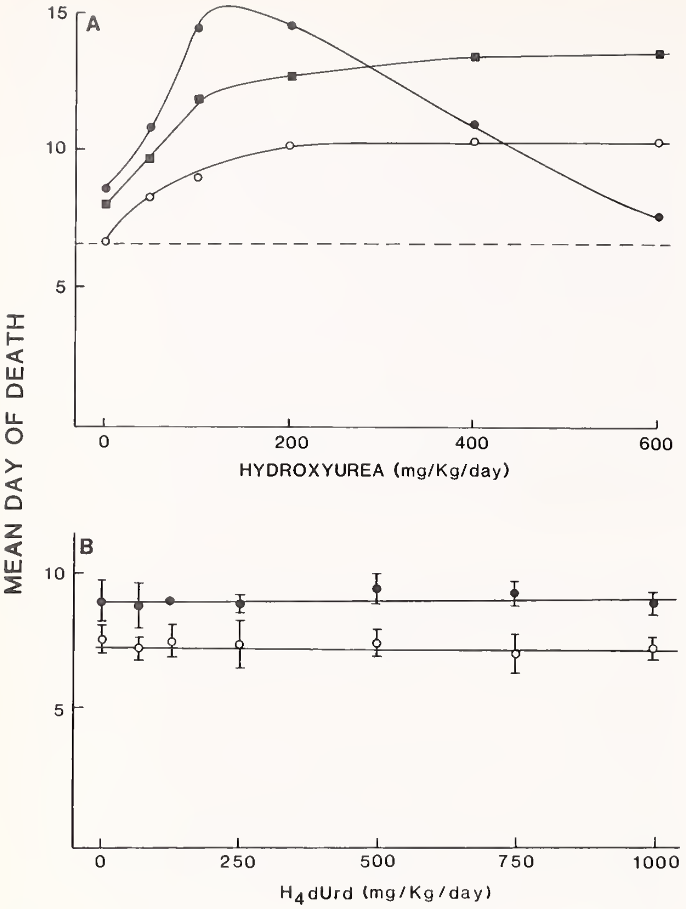


FIGURE 3.—Treatment of mice bearing the L1210 mouse leukemia with combinations of FUdR and inhibitors of deoxyuridylate synthesis. DBA/2 female mice (10 animals per group) were inoculated ip with 10^6 leukemic cells on day zero and were subsequently treated with drugs ip. A) Mice were treated with either HU alone (○) or in combination with FUdR at 75 mg/kg/day (●) or 40 mg/kg/day (■). HU was administered daily until the death of the animals starting on day 1. FUdR was given on days 1–5, 8, 11, and 14. B) Mice were treated with H_4dUrd at the indicated doses daily for 9 days starting on day zero, either alone (○) or in combination with 75 mg/kg/day of FUdR (●) administered on days 1–5. *Dotted line* shows the survival of phosphate-buffered saline-treated control animals. Reprinted with permission from Biochem Pharmacol 31:2929–2935, 1982.

the treatment of the L1210 mouse leukemia in vivo. A therapeutic synergism was found with the coadministration of FUdR and either HU or GZ (*29*) (fig. 3A). The administration of substantial doses of H_4dUrd was without effect on this tumor by itself and H_4dUrd did not increase the utility of FUdR (fig. 3B). The most effective treatment of this series was found to be FUra combined with GZ, which extended the lifespan of DBA-2 female mice, inoculated with 10^6 cells, by 15 days beyond the mean day of death of controls. The logical extension of these studies would be the evaluation of the combined use of FUra, folinic acid and inhibitors of ribonucleotide reductase as a therapeutic modality. However, the timing of the administration of ribonucleotide reductase inhibitors might be critical in such studies because both uracil and fluorouracil ribonucleotides are reduced to deoxyribonucleotides. If scheduling is required to block dUMP accumulation without affecting the synthesis of FdUMP, it is most likely that drugs such as HU should follow FUra by 6 to 12 hours.

REFERENCES

(*1*) HEIDELBERGER C: *In* Antineoplastic and Immunosuppressive Agents (Sartorelli AC, Johns DG, eds). New York: Springer-Verlag, 1975, pp 193–231.

(*2*) LIVINGSTONE RB, CARTER S: Single Agents in Cancer Chemotherapy. New York: Plenum Press, 1970.

(*3*) ENSMINGER WD, ROSOWSKY A, RASO V, et al: A clinical-pharmacological evaluation of hepatic arterial infusions of 5-fluoro-2′-deoxyuridine and 5-fluorouracil. Cancer Res 38:3784–3792, 1978.

(*4*) BURCHENAL JH, HOLBERG EA, FOX JJ, et al: The effects of 5-fluorodeoxycytidine, 5-fluorodeoxyuridine and related compounds on transplanted mouse leukemias. Cancer Res 19:494–500, 1959.

(*5*) HEIDELBERGER C, GRIELACH L, MONTAG BJ, et al: Studies on fluorinated pyrimidines. II. Effects on transplanted tumors. Cancer Res 18:305–317, 1958.

(*6*) CARTER SK, GOLDIN A: Experimental models and their clinical correlations. Natl Cancer Inst Monogr 45:63–74, 1977.

(*7*) HARTMANN JR, ORIGENES ML, JR, MURPHY ML: Effects of 2′-deoxy-5-fluorouridine (NSC-27640) and 5-fluorouracil (NSC-19893) on childhood leukemia. Cancer Chem Rep 34:51–54, 1964.

(*8*) LAND VJ, DYMENT PG, KOMP D, et al: 5-Fluorouracil (NSC-19893) and actinomycin-D (NSC-3053) in the treatment of acute leukemia in children. Cancer Chemother Rep 57:335–339, 1973.

(*9*) HOUGHTON JA, MARODA SJ, PHILLIPS JO, et al: Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenographs of human colorectal adenocarcinomas in mice. Cancer Res 41:144–149, 1981.

(*10*) ULLMAN B, MELINDA L, MARTIN DW, et al: Cytotoxicity of 5-fluoro-2′-deoxyuridine requirement for reduced folate cofactors and antagonism by methotrexate. Proc Natl Acad Sci USA 75:980–983, 1978.

(*11*) EVANS RM, LASKIN JD, HAKALA MT: Effects of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. Cancer Res 41:3288–3295, 1981.

(*12*) AYUSAWA D, KOYAMA H, IWATA K, et al: Single step selection of mouse FM3A cell mutants defective in thymidylate synthetase. Somatic Cell Mol Genet 6:261–270, 1980.

(*13*) KEYOMARSI K, MORAN RG: Folinic acid augmentation of the effects of fluoropyrimidines on murine and human leukemic cells. Cancer Res 46:5229–5235, 1986.

(*14*) MORAN RG, COLMAN PD: A simple procedure for the synthesis of high specific-activity tritiated (6-S)5-formyltetrahydrofolate. Anal Biochem 122:70–78, 1982.

(*15*) HEIDELBERGER C, DANENBERG PV, MORAN RG: Fluoropyrimidines and their nucleosides. Adv Enzymol 54:57–119, 1982.

(*16*) DANENBERG PV, DANENBERG KD: Effect of 5,10-methylenetetrahydrofolate and the dissociation of 5-fluoro-2′-deoxyuridylate from thymidylate synthetase: Evidence for an ordered mechanism. Biochemistry 17:4018–4024, 1978.

(*17*) LOCKSHIN A, DANENBERG PV: Biochemical factors affecting the tightness of 5-fluorodeoxyuridylate binding of human thymidylate synthetase. Biochem Pharmacol 30:247–257, 1980.

(*18*) KLUBES P, CONNELLY K, CERNA J, et al: Effects of 5-fluorodeoxyuridine-5′-monophosphate and 2′deoxyuridine-5′-monophosphate pools, and DNA synthesis in solid mouse L1210 and rat Walker 256 tumors. Cancer Res 38:2325–2331, 1978.

(*19*) MORAN RG, SPEARS CP, HEIDELBERGER C: Biochemical determinants of tumor sensitivity to 5-fluorouracil. Ultrasensitive method for the determination of 2′-deoxyuridylate, and thymidylate synthetase. Proc Natl Acad Sci USA 76:1456–1460, 1979.

(*20*) Meyers CE, Young RC, Johns DG, et al: Assay of 5-fluorodeoxyuridine-5′-monophosphate pools following 5-fluorouracil. Cancer Res 34:2682–2688, 1974.

(*21*) Myers CE, Young RC, Chabner BA: Biochemical determinants of 5-fluorouracil response in vivo. The role of deoxyuridylate pool expansion. J Clin Invest 56:1231–1238, 1975.

(*22*) Grindey GB, Moran RG, Werkheiser WC: Approaches to the rational combination of antimetabolites for cancer chemotherapy. Adv Drug Design 5:170–249, 1975.

(*23*) Moran RG, Zakrzewski SF, Werkheiser WC: Folate metabolism in mammalian cells in culture. I. Partial characterization of the folates present in mouse L1210 leukemia cells. J Biol Chem 251:3569–3575, 1976.

(*24*) Priest DG, Happel KK, Mangum M, et al: Tissue folylpolyglutamate chain length characterization by electrophoresis as thymidylate synthetase-fluorodeoxyuridylate ternary complexes. Anal Biochem 115:163–169, 1981.

(*25*) Ardalan B, Glazer RI, Kensler TW, et al: Synergistic effect of 5-fluorouracil and *N*-(phosphoracetyl)-L-aspartate on cell growth and ribonucleic acid synthesis in a human mammary carcinoma. Biochem Pharmacol 30:2045–2049, 1981.

(*26*) Kufe DW, Egan EM: Enhancement of 5-fluorouracil incorporation into human lymphoblastic ribonucleic acid. Biochem Pharmacol 30:129–132, 1981.

(*27*) Meshad M, Ervin T, Kufe D, et al: Phase I trial of combination therapy with PALA and 5-FU. Cancer Treat Rep 65:331–336, 1981.

(*28*) Maley F, Maley GF: Tetrahydrodeoxyuridylate: A potent inhibitor of deoxycytidylate deaminase. Arch Biochem Biophys 144:723–729, 1971.

(*29*) Moran RG, Danenberg PV, Heidelberger C: Therapeutic response of leukemic mice treated with fluorinated pyrimidines and inhibitors of deoxyuridylate synthesis. Biochem Pharmacol 31:2929–2935, 1982.

Biochemical and Pharmacologic Basis for Potentiation of 5-Fluorouracil Action by Leucovorin[1]

Y. M. Rustum,* F. Trave, S. F. Zakrzewski, N. Petrelli, L. Herrera, A. Mittelman, S. G. Arbuck, and P. J. Creaven[2,3]

ABSTRACT—In vitro and in vivo studies have been carried out in mouse and human tumors to investigate the biochemical and pharmacologic basis for the selectivity of 5-fluorouracil (FUra) action. Combination chemotherapy with FUra and thymidine was performed to determine the therapeutic relevance of 5-fluorouridine triphosphate (FUTP) incorporation into RNA. The results of these studies indicate that modulation of FUra cytotoxicity by deoxythymidine (dThd) did occur but failed to produce any significant therapeutic advantages in patients with advanced colorectal cancer. Modulation of FUra bioactivation via the deoxyribonucleotide pathway by coadministration of high-dose folinic acid resulted in enhanced therapeutic response rate of gastrointestinal tumor patients. This manuscript summarizes the preclinical and clinical findings on the metabolic modulation of FUra activity by dThd and folinic acid.—NCI Monogr 5:165–170, 1987.

FUra is a fluorinated pyrimidine widely used in the treatment of a variety of solid tumors (*1*). FUra is cytotoxic to eukaryotic cells mainly through bioactivation to 5-fluorodeoxyuridine monophosphate (FdUMP), which is a potent inhibitor of thymidylate synthase (dTMP-S), and leads to inhibition of DNA synthesis (*2–5*) and to FUTP, which, upon incorporation into RNA, can result in nonfunctional ribonucleic acids (*6–9*). The metabolic pathways involved in FUra bioactivation are outlined in figure 1.

It has been demonstrated in vitro in several established cell lines of mouse and human origin that incorporation of FUTP into RNA can be the lethal event in some tumor cell lines, while inhibition of DNA synthesis seems to be the growth-limiting event of FUra action in other cell lines (*10–12*). During the last few years, attempts have been made to identify determinants of response to FUra in human tumor cells and to determine if there is a relationship between biochemical characteristics of target cells and response (*5,11*). Determinants that have been evaluated include measurement of the activity of key enzymes responsible for FUra metabolic activation such as dThd phosphorylase and kinase, uridine (Urd) phosphorylase and kinase, phosphoribosyl pyrophosphate (PRPP) transferase, and dTMP synthase (*10,11,13*). The results of enzyme studies carried out in Dr. Hakala's laboratory can be summarized as follows: Cells that are less sensitive to FUra tend to have lower PRPP transferase activity and higher dThd kinase activity than more sensitive cells (*10*).

The therapeutic relevance of FUra incorporation into RNA has been investigated in a number of systems with conflicting results. Coadministration of dThd and FUra will produce the following alterations: 1) reduced conversion rate of 5-fluorodeoxyuridine (FdUrd) into FdUMP due to dThd-FdUrd competition for dThd kinase, and 2) reduced conversion of 5-fluorouridine diphosphate (FUDP) into 5-fluorodeoxyuridine diphosphate (FdUDP) due to dTTP feedback inhibition of ribonucleotide reductases (*14*). Under these conditions, FUra bioactivation will be "forced" in the direction of the ribonucleotide pathway. Martin and Stolfi (*12,15*) showed that modulation of FUra cytotoxicity by dThd could increase the therapeutic index of the antimetabolite in mice bearing breast tumors and demonstrated that this effect was due primarily to the observed increase of FUra incorporation into RNA. Furthermore, Laskin et al. (*10*) showed that the growth inhibition of most human tumor cells when exposed in vitro to FUra was caused mainly by FUra incorporation into RNA. The combination of dThd and FUra was therefore investigated in colon tumor model systems (*16,17*) and in patients with advanced colorectal carcinoma (*18,19*). The result of these investigations showed no significant improvement on the therapeutic efficacy of FUra either in colon model systems or in patients with advanced colorectal carcinoma.

The importance of DNA synthesis inhibition to the therapeutic activity of FUra has also been investigated. Hakala and coworkers reported that in vitro growth inhibition by FUra in human Hep-2 cells increased 3-fold if an excess of folinic acid (D,L-CF) was added to the culture medium (*20*). The concentration of D,L-CF required to gain the 3-fold increase of FUra-mediated growth inhibition was 20 μM (by 24-hr exposure to D,L-CF prior to a 3-hr exposure to FUra). This potentiation of FUra cytotoxicity by

---

ABBREVIATIONS: $CH_2FH_4 = N^{5,10}$ methylenetetrahydrofolic acid; $5\text{-}CH_3FH_4$ = 5-methyltetrahydrofolic acid; D,L-CF = D5L-5-formyltetrahydrofolic acid, folinic acid, leucovorin; dThd = deoxythymidine; dTMP-S = thymidylate synthase; dTTP = deoxythymidine triphosphate; FdUDP = 5-fluorodeoxyuridine diphosphate; FdUMP = 5-fluorodeoxyuridine monophosphate; FdUrd = 5-fluorodeoxyuridine; FUDP = 5-fluorouridine diphosphate; FUTP = 5-fluorouridine triphosphate; ID_{50} = concentration required for 50% inhibition of growth; PRPP = phosphoribosyl pyrophosphate; $t\frac{1}{2}$ = half-life; Urd = uridine.

[1] Supported in part by Program Grant CA-21071, Project Grant CA-18420, and Core Grant CA-24538.

[2] Roswell Park Memorial Institute, Buffalo, NY.

[3] We thank C. Frank, J. Goranson, and P. Recht for technical assistance, and C. Melancon and G. Wagner for their help in typing this manuscript.

* *Reprint requests to:* Y. M. Rustum, Ph.D., Grace Cancer Drug Center, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263.

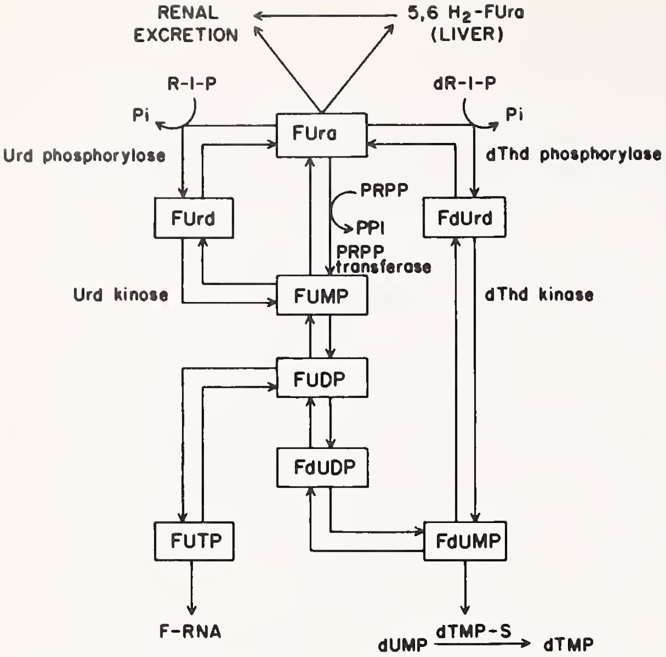


FIGURE 1.—Metabolism of FUra. dR-1-P=deoxyribose-1-phosphate; P_i=inorganic phosphate; PP_i=inorganic pyrophosphate; R-1-P=ribose-1-phosphate.

D,L-CF resulted from increased intracellular dTMP-S cofactor pool (*20,21*) and consequently delayed recovery of dTMP-S activity (*22*). These results demonstrate that significant potentiation of FUra action could be achieved in cells when the intracellular folate pool was increased by administration of exogenous folates. From a biochemical point of view, the coadministration of D,L-CF shifted the critical site of action of FUra in human cells from an RNA effect to a DNA effect. If such a modulation can also be achieved in vivo, the combination of FUra and D,L-CF may be therapeutically useful in tumors which have become resistant to FUra, when resistance is due to the presence of inadequate intracellular levels of dTMP-S cofactor.

In this paper, specific approaches to the modulation of FUra activation will be reviewed. The biochemical and pharmacologic basis for the observed effects of modulators such as dThd and D,L-CF on the activity of FUra will be discussed.

RESULTS AND DISCUSSION

Modulation of FUra by dThd

Preclinical Studies

Several human and mouse tumor cell lines were tested in vitro for their sensitivity to growth inhibition by FUra. The median concentration required for 50% inhibition of growth (ID_{50}) of human lines for FUra was 2.8 μM, while mouse cell lines displayed a median ID_{50} of only 0.4 μM (*10*). Thus, most human cell lines were less sensitive to FUra than most mouse cell lines. While the main site of action of FUra in mouse cell lines was inhibition of dTMP-S, in human cells incorporation of FUra into RNA was the primary cause of cellular growth inhibition (*11*). This conclusion is based on the observation that only mouse cells could be rescued from FUra toxicity by dThd.

The in vitro incorporation of FUra into RNA and the formation of FdUMP were studied in human colon tumor cells following 1-hour incubation with FUra alone or preceded by a 3-hour incubation with dThd (*18*). The results, which are summarized in table 1, clearly demonstrate the inhibitory effects on FdUMP formation by dThd. There was a greater than 4-fold decrease of free FdUMP in the acid-soluble fraction, with concomitant enhancement of FUra-RNA formation.

In vivo studies in rodents bearing colon carcinoma were carried out to investigate the potential therapeutic synergism of the combination of dThd and FUra (*16,17*). These studies showed that dThd did not enhance the antitumor efficacy of FUra at equitoxic doses against mouse colon 26, colon 38 carcinomas (*16*), or against a dimethylhydrazine-induced rat colon carcinoma (*17*). If equimolar doses were chosen, however, the combination of dThd and FUra produced more tumor-free survivors than did the antimetabolite alone (*17*).

Clinical Studies

Concurrent infusion of dThd and FUra was administered to patients with colorectal carcinoma. The presence of dThd did alter the pharmacokinetics (*18*) and the toxicity profile (*19*) of FUra, but the response rate for the dThd-FUra combination was not significantly different from that observed with FUra alone (*19*).

The in vitro studies of the rescue of FUra-treated cells with dThd provided valuable information about the mechanisms of action of FUra in various tumor cell lines in culture. Results in vivo showed that, at least in colon carcinoma, the combination of FUra and dThd failed to produce significant improvement of the therapeutic index of FUra. These results suggest that incorporation of FUra into RNA may be more related to host toxicity than to efficacy in patients with advanced colorectal cancers.

Modulation of FUra by D,L-CF

Preclinical Studies

To investigate the basis of the observed differences in FUra activity between human and mouse cell lines in culture, two representative tumor lines, human Hep-2 carcinoma cells and mouse S-180 sarcoma cells, were extensively studied (*20–23*). Since dTMP-S inhibition was the critical site of the FUra cytotoxicity in S-180 cells but not in Hep-2 cells, the pools of FdUMP and dUMP were quantitated before and at various times after a 3-hour exposure to different FUra concentrations (*22*). A parallel increase of FdUMP and dUMP pools was observed with increasing

TABLE 1.—In vitro effect of dThd on FUra metabolism in human colon carcinoma cells[a]

Treatment[b]	Free FdUMP in the acid-soluble fraction, pmol/10^7 cells[c]	FUra incorporation into RNA, pmol/10^7 cells[c]
FUra	1.2	12
dThd-FUra	<0.3	50

[a] Reprinted with permission from Cancer Res 42:2930–2937, 1982.

[b] Cells were treated with 100 μM dThd for 3 hr, incubated with labeled FUra (7.7 μM) for 1 hr and extracted for quantitation of free FdUMP and for evaluation of FUra incorporation into RNA.

[c] Median values.

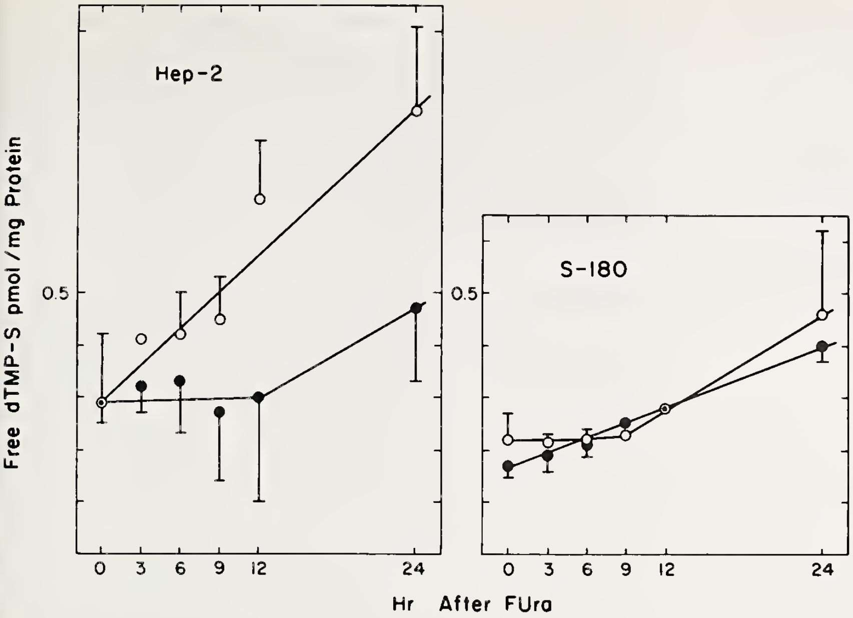


FIGURE 2.—Effect of D,L-CF in combination with FUra on the recovery of dTMP-S. Cells were preincubated in medium with (●) or without (○) 20 μM D,L-CF for 24 hr. Incubation was then continued in presence of FUra for 3 hr followed by drug-free medium for another 24 hr. Cells were then evaluated at various times for their dTMP-S content. Reprinted with permission from Mol Pharmacol 25:303–309, 1984.

concentrations of FUra in both cell lines, but with a more pronounced increase in Hep-2 cells (the less FUra-sensitive cell line). Human Hep-2 cells accumulated more FdUMP than the highly sensitive mouse S-180 cells. These results strongly suggested that FdUMP is an essential but not sufficient effector of drug response. Furthermore, the difference in sensitivity between mouse and human cells in culture could not be explained solely on the basis of differences in degree of dTMP-S inhibition. It is possible that differences in tumor sensitivity to fluoropyrimidines may lie in the duration of inhibition of DNA synthesis, which is a function of the stability of the FdUMP–dTMP-S-folate cofactor ternary complex. Therefore, studies were carried out to quantitate the intracellular concentration of the dTMP-S cofactor, $N^{5,10}$methylenetetrahydrofolic acid (CH_2FH_4), in cell lines with different sensitivity to FUra and to evaluate the consequence of manipulation of these pools (*20–23*). The results indicated that the “basal” content of CH_2FH_4 is more than twice as high in S-180 cells than in Hep-2 cells. When 20 μM D,L-CF was added to the growth medium, the intracellular concentration of CH_2FH_4 showed a parallel increase in S-180 and Hep-2 cells. In addition, the ID_{50} for a 3-hour exposure to FUra decreased by approximately 60%. Unlike mouse S-180 cells, human Hep-2 cells appear to have preexisting pools of CH_2FH_4 that are inadequate for optimal stabilization of the ternary complex; exogenous folates must be provided to increase the intracellular folate pools and thereby enhance the stability of the FdUMP-dTMP-S-CH_2FH_4 complex.

Further evidence for the importance of stabilization of the ternary complex in cytotoxicity of fluoropyrimidines is shown in figure 2, which illustrates the effect of D,L-CF in combination with FUra on the spontaneous recovery of dTMP-S activity in S-180 and Hep-2 cell lines (*22*). Delay in dTMP-S recovery was observed only in Hep-2 cells. In the presence of 20 μM D,L-CF, the recovery of enzyme activity became an important determinant for growth inhibition of Hep-2 cells in culture. These data strongly suggest that in the presence of D,L-CF, the primary mode of action of FUra is the prolonged inhibition of dTMP-S.

Studies to evaluate the intracellular concentrations of CH_2FH_4 pools in different mouse tumors have been initiated. Preliminary results are shown in table 2. These data indicate that the levels of dTMP-S cofactor pools vary significantly among tumors of different types. In FUra sensitive cells such as L1210 and P-1798, dTMP-S cofactor levels are significantly higher than those observed in vivo in other less sensitive cell lines.

To determine if there is a relationship between plasma levels of D,L-CF, L-CF and 5-CH_3FH_4 and the intracellular CH_2FH_4 pools, studies have been initiated in mice bearing L1210 leukemia cells. Preliminary results are summarized in table 3. These findings suggest that in L1210 (with relatively high levels of pre-existing folate cofactor, see table 2) it is possible to modulate the intracellular CH_2FH_4 when D,L-CF is administered by 24-hour infusion at doses corresponding to about 150 to 600 mg/m^2 in man. The consequences of such in vivo modulation on the therapeutic efficacy of FUra and FdUrd against mice bearing transplantable tumors is under investigation.

Clinical Studies

Colorectal cancer

Results in cell culture demonstrated that continuous exposure for 24 hours to 20 μM D,L-CF (10 μM L-CF) was optimal for stabilization of the ternary complex and for

TABLE 2.—Intracellular CH_2FH_4 pools in different tumors

Tumor type	dTMP-S cofactor, pmol/mg protein In culture	 In mice
Mouse leukemia L1210	32.2	80.0
Mouse melanoma B16	24.8	9.1
Mouse colon carcinoma 38	—[a]	32.5
Mouse colon carcinoma 28	—[a]	8.3
Mouse lymphoma P1798	—[a]	129.2
Human colon carcinoma 205	13.3	8.9

[a] Not determined.

TABLE 3.—Relationship between plasma folates and intracellular CH_2FH_4 pools in mice bearing L1210 cells

D,L-CF dose, mg/kg/day	Plasma concentration, μM			dTMP-S cofactor pool in L1210 cells, pmol cofactor/mg protein
	D,L-CF	L-CF	$5\text{-}CH_3FH_4$	
None	—[a]	—[a]	—[a]	79
50	5.8	0.2	<0.5	158
200	9.5	0.6	0.9	320
400	17.1	1.0	2.6	—[a]
800	39.4	3.8	4.6	—[a]

[a] Not determined.

potentiation of the cytotoxic effects of FUra (*20–23*). On the basis of these results, a clinical phase I–II protocol of FUra in combination with high-dose D,L-CF was designed to evaluate the toxicity and therapeutic efficacy of this combination in patients with advanced colorectal cancers (*24*). As part of this study, plasma pharmacokinetics of folates and FUra (*25,26*) and, whenever possible, studies on the inhibition and recovery of dTMP-S activity in the patients' tumors were carried out (*26*).

The schedule of treatment was as follows: D,L-CF (500 mg/m^2) by 2-hour iv infusion + FUra by iv push, 60 minutes after the start of the D,L-CF infusion. The FUra starting dose was 300 mg/m^2, with escalation to toxicity in the phase I trial. The recommended dose for phase II and III was 600 mg/m^2. The D,L-CF dose of 500 mg/m^2 was chosen to achieve L-CF plasma levels greater than 10 μM.

Plasma pharmacokinetics indicated that the concentration of L-CF remained above 10 μM for approximately 2 hours. L-CF clearance from plasma was relatively rapid (mean elimination half-life (t½) was 1.2 hr). Plasma levels of $5\text{-}CH_3FH_4$ remained above 10 μM for a period of more than 6 hours, with a slow plasma clearance (mean elimination t½ was 7.0 hr). These results indicated that greater than 10 μM total active folate was achieved and maintained in patient plasma for several hours after termination of D,L-CF treatment. The effect of the high folate plasma levels on the pharmacokinetics of FUra was also investigated (*25*). FUra elimination half-life increased by 26% in the group of patients with high folate plasma levels (9.6 ± 0.8 min vs. 12.1 ± 2.2 min; $P < 0.05$), and FUra plasma clearance decreased from 16.4 to 14.0 ml/kg × minutes. A possible explanation for this change in FUra pharmacokinetics could be an altered FUra metabolism.

The overall response rate in this study approached 40%. In the phase I study, 6 of 12 patients previously resistant to FUra alone did respond to the combination of FUra + D,L-CF (*24*). Preliminary results also indicate a relationship between response and duration of inhibition of dTMP-S in the tumor tissue (*26*).

In summary, clinical, biochemical and pharmacological data indicate that the metabolic modulation of FUra can be achieved in man with high dose D,L-CF and that this modulation increases the therapeutic efficacy of FUra in patients with advanced colorectal carcinoma when optimal conditions identified in the model system are achieved clinically.

Gastric cancer

A phase II study of the combination of FUra and high-dose D,L-CF in patients with advanced gastric cancers is ongoing at Roswell Park. Of 20 patients evaluated for response at the time of this report, 11 had progressed previously on FUra-containing regimens. Three patients had partial responses, including 2 who had previously received FUra. Nine patients had stable disease and 7 progressed. One heavily pretreated patient died of toxicity after 4 treatments. Additional patients and longer follow-up will be required to determine the usefulness of this regimen in patients with gastric carcinoma.

Gastrointestinal tumors confined primarily to the abdomen

The clinical feasibility and pharmacokinetic advantage of a FUra intraperitoneal chemotherapy in man has been demonstrated (*27,28*). The combination of ip FUra and D,L-CF was studied in a phase I trial at Roswell Park (*29,30*). Patients received 8 consecutive 4-hour exchanges consisting of 2 liters of peritoneal dialysis fluid containing the 2 drugs. The starting FUra dose of 2.0 mM was escalated by 0.5-mM increments until the maximally tolerated dose (4.0 mM) was identified. In an attempt to achieve a L-CF concentration of 10 μM, 20.8 μM of D,L-CF was administered in each exchange. FUra was detected in the plasma even at the lowest dose employed (2.0 mM), thus providing a second route of drug delivery via the systemic

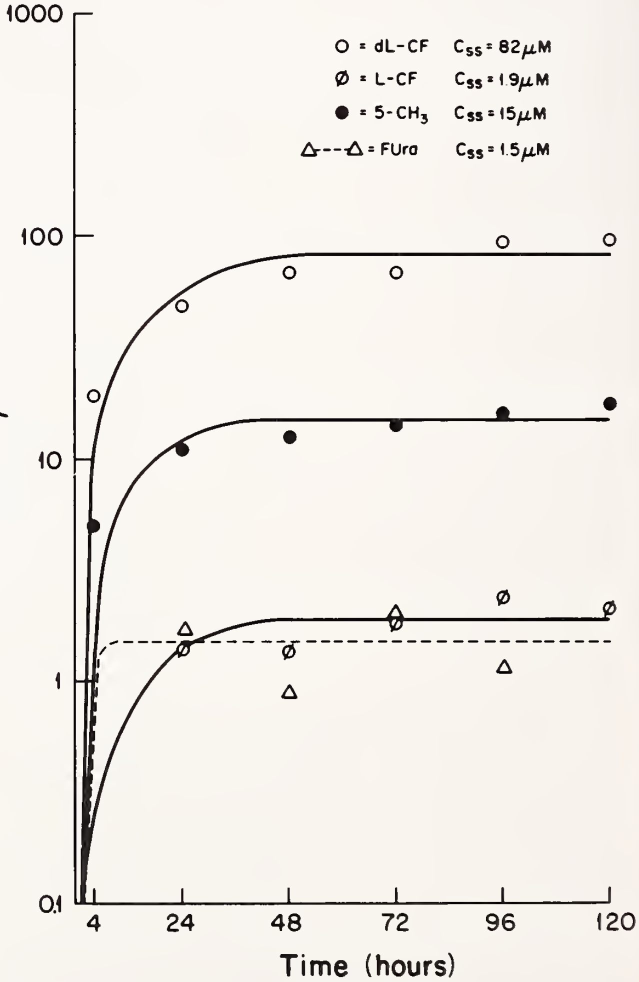


FIGURE 3.—Plasma profile of FUra (600 mg/m^2/day for 5 days) and folates (D,L-CF; 500 mg/m^2/day for 5 days) in 1 patient with metastatic colorectal carcinoma. C_{ss} = concentration at steady state.

circulation. At the 20.8-μM dose of D,L-CF, plasma levels of D,L-CF and 5-CH_3FH_4 were below the detection limit of the assay (0.5 μM). L-CF levels in the peritoneal cavity were approximately 3.0 μM after 1 hour and less than 1.5 μM at 4 hours. For these reasons, the dose of D,L-CF in the first exchange was escalated to 104 μM. Clinical results of this phase I trial are reported elsewhere in this journal.

Future Directions

The rapid plasma clearance of FUra, its phase-specificity, and the relatively short elimination of L-CF (*25,26,31*) are sufficient rationale for the evaluation of these agents by continuous iv infusion. Based on these pharmacologic considerations and on the encouraging results of clinical trials to date, a treatment protocol with the combination of FUra and high-dose D,L-CF administered by 5-day continuous iv infusion was initiated recently at Roswell Park. The plasma profile of FUra and folates in one patient is shown in figure 3. Together with the pharmacokinetics, the evaluation of the dTMP-S activity in the tumor tissue before and daily during the 5 days of treatment is being performed whenever possible. Preliminary results indicate that inhibition of dTMP-S occurred only during the first 2 days of therapy. If these results are confirmed, the following questions can be asked: 1) Is the lack of prolonged inhibition of dTMP-S due to inability to modulate the effects of FUra at the cellular level? 2) Is there a relationship among the steady-state concentrations of L-CF and 5-CH_3FH_4, intracellular folate cofactor pools and response? 3) If the steady-state plasma concentration of active folates is not sufficient to modulate FUra effects, are these folate concentrations sufficient to modulate FdUrd efficacy? Preclinical and clinical investigations are under way in this laboratory to evaluate these concepts and to document optimal conditions for the use of D,L-CF in combination with FUra or FdUrd.

REFERENCES

(*1*) De Vita VT, Jr, Hellman S, Rosenberg A, (eds): Cancer—Principles and Practice of Oncology. Philadelphia: J. B. Lippincott Co., 1982.

(*2*) Hartman KU, Heidelberg C: Studies on fluorinated pyrimidines. XIII. Inhibition of thymidylate synthetase. J Biol Chem 236:3006–3013, 1961.

(*3*) Santi DV, McHenry CS, Sommer H: Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. Biochemistry 13:471–480, 1974.

(*4*) Danenberg PV, Locksin A: Fluorinated pyrimidines as tight-binding inhibitors of thymidylate synthetase. Pharmacol Ther 13:69–90, 1981.

(*5*) Houghton JA, Maroda SJ, Phillips JD, et al: Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinoma in mice. Cancer Res 41:144–149, 1981.

(*6*) Mandel HG: The incorporation of 5-fluorouracil in RNA and its molecular consequences. Prog Mol Subcell Biol 1:82–135, 1969.

(*7*) Glazer RI, Peale AL: The effect of 5-fluorouracil on the synthesis of nuclear RNA in L1210 cells in vitro. Mol Pharmacol 16:270–277, 1979.

(*8*) Kufe SW, Major PP: 5-Fluorouracil incorporation into human breast carcinoma DNA correlates with cytotoxicity. J Biol Chem 256:9802–9805, 1981.

(*9*) Glazer RI, Lloyd LS: Association of cell lethality with incorporation of 5-fluorouracil and 5-fluorouridine into nuclear RNA in human colon carcinoma cells in culture. Mol Pharmacol 21:468–473, 1982.

(*10*) Laskin JD, Evans RM, Slocum HK, et al: Basis for natural variation in sensitivity to 5-fluorouracil in mouse and human cells in culture. Cancer Res 39:383–390, 1979.

(*11*) Evans RM, Laskin JD, Hakala MD: Assessment of growth limiting events caused by 5-fluorouracil in mouse cells and in human cells. Cancer Res 40:4113–4122, 1980.

(*12*) Martin DS, Stolfi RL: Thymidine enhancement of antitumor activity of 5-fluorouracil against advanced murine breast carcinoma. Proc Am Assoc Cancer Res, ASCO 18:126, 1977.

(*13*) Peters JA, Laureusse E, Leyua A, et al: Sensitivity of human, murine and rat cells to 5-fluorouracil and 5′-deoxy-5-fluorouridine in relation to drug metabolizing enzymes. Cancer Res 46:20–28, 1986.

(*14*) Moore EC, Hurlbert RB: Regulation of mammalian deoxyribonucleotide biosynthesis by nucleotides as activators and inhibitors. J Biol Chem 241:4802–4809, 1966.

(*15*) Martin DS, Stolfi RL, Spiegelman S: Striking augmentation of the in vivo anticancer activity of 5-fluorouracil by combination with pyrimidine nucleosides: An RNA effect. Proc Am Assoc Cancer Res, ASCO 19:221, 1978.

(*16*) Danhauser LL, Rustum YM: A method for continuous drug infusion in unrestrained rats: Its application in evaluating the toxicity of 5-fluorouracil/thymidine combination. J Lab Clin Med 93:1047–1053, 1979.

(*17*) Danhauser LL, Rustum YM: Chemotherapeutic efficacy of 5-fluorouracil with concurrent thymidine infusion against transplantable colon tumor in rodents. Cancer Drug Deliv 1:269–282, 1984.

(*18*) Au JL-S, Rustum YM, Ledesma EJ, et al: Clinical pharmacological studies of concurrent infusion of 5-fluorouracil and thymidine in treatment of colorectal carcinomas. Cancer Res 42:2930–2937, 1982.

(*19*) Sternberg A, Petrelli N, Au J, et al: A combination of 5-fluorouracil and thymidine in advanced colorectal carcinoma. Cancer Chemother Pharmacol 13:218–222, 1984.

(*20*) Evans RM, Laskin JD, Hakala MT: Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. Cancer Res 41:3288–3295, 1981.

(*21*) Yin M-B, Zakrzewski SF, Hakala MT: Relationship of cellular folate cofactor pools to activity of 5-fluorouracil. Mol Pharmacol 23:190–197, 1984.

(*22*) Berger SE, Hakala MT: Relationship of dUMP and free FdUMP pools to inhibition of thymidylate synthase by 5-fluorouracil. Mol Pharmacol 25:303–309, 1984.

(*23*) Hakala MT: Basis for the effect of leucovorin on the activity and site of action of 5-fluorouracil in human cells. *In* Advances in Cancer Chemotherapy—The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: J. Wiley & Sons, Inc., 1984, pp 33–39.

(*24*) Madajewicz S, Petrelli N, Rustum Y, et al: Phase I–II trial of high-dose calcium leucovorin and 5-fluorouracil in advanced colorectal cancer. Cancer Res 44:4667–4669, 1984.

(*25*) Trave F, Rustum YM, Mazzoni A, et al: Possible effect of high dose 5-formyltetrahydrofolic acid on the pharmacokinetics of 5-fluorouracil in patients with colorectal carcinoma. Proc ASCO 4:30, 1985.

(*26*) Rustum YM, Madajewicz S, Campbell J, et al: Clinical biochemical pharmacology of 5-formyltetrahydrofolate in combination with 5-fluorouracil in patients with advanced colorectal carcinoma: Correlation with clinical response. *In* Advances in Cancer Chemotherapy—The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination

(Bruckner HW, Rustum YM eds). New York: J. Wiley & Sons, Inc., 1984, pp 77–82.

(*27*) Speyer JL, Collins JM, Dedrick RL, et al: Phase I and pharmacological studies of 5-fluorouracil administered intraperitoneally. Cancer Res 40:567–572, 1980.

(*28*) Ozols RF, Spayer JE, Jenkins J, et al: Phase II trial of 5-FU administered i.p. to patients with refractory ovarian cancer. Cancer Treat Rep 68:1229–1232, 1984.

(*29*) Arbuck SG, Trave F, Douglass HO, et al: A phase I trial of intraperitoneal (IP) 5-fluorouracil (FU) and folinic acid (FA) in gastrointestinal (GI) tumors. Proc Am Assoc Cancer Res 27:172, 1986.

(*30*) Arbuck SG, Trave F, Douglass HO Jr, et al: Phase I and pharmacological studies of intraperitoneal leucovorin and 5-fluorouracil in patients with advanced cancers. J Clin Oncol 4:1510–1517, 1986.

(*31*) Straw JA, Szapary D, Winn WT: Pharmacokinetics of the diastereoisomers of leucovorin after intravenous and oral administration to normal subjects. Cancer Res 44:3114–3119, 1984.

Preliminary Analysis of a Randomized Comparison of 5-Fluorouracil Versus 5-Fluorouracil and High-Dose Continuous-Infusion Folinic Acid in Disseminated Colorectal Cancer[1]

James H. Doroshow,* Marcelle Bertrand, Edward Newman, Phyllis Multhauf, Lucille Leong, Douglas Blayney, David Goldberg, Kim Margolin, Brian Carr, Stephen Akman, and Gerald Metter[2,3]

ABSTRACT—**In this study, 50 patients were randomly assigned to treatment with 5-fluorouracil (FUra) or FUra plus high-dose continuous-infusion folinic acid. Five of 27 evaluable patients in the FUra group versus 10 of 21 patients in the FUra plus folinic acid arm of the study had objective partial remissions, $P = 0.02$. Time to progression was 3.9 months for FUra and 8.0 months for FUra and folinic acid, $P = 0.006$; however, median survivals (11.9 versus 14.5 months) were not different in this crossover study. Toxicity in both treatment arms was mild, although patients receiving FUra plus folinic acid experienced significantly more stomatitis than patients treated with FUra alone. This study suggests that high-dose, continuous-infusion folinic acid, which produces a steady-state level of biologically active folates of 10 μM, significantly increases the therapeutic activity of FUra.—NCI Monogr 5:171-174, 1987.**

In recent years, there have been many attempts to enhance the therapeutic utility of FUra in gastrointestinal malignancies by modification of its intracellular metabolism (*1,2*). Based on preclinical studies, modulation of fluoropyrimidine action by methotrexate (*2*), thymidine (*3*), or *N*-(phosphonacetyl)-L-aspartic acid (*1,4*) has been tested in the clinic with variable success. Because of experiments from several laboratories indicating that pharmacologic concentrations of reduced folates in vitro could enhance the degree of thymidylate synthase inhibition produced by FUra (*5-7*), investigators have recently suggested that the combination of folinic acid and FUra produces therapeutic synergy in the treatment of human colorectal and gastric cancer (*8,9*). Because the uptake of reduced folate cofactors by certain human carcinoma cell lines is slow, requiring, under some circumstances, 8 to 12 hours to reach half-maximal levels of intracellular folates (*7*), we undertook a prospective, randomized trial evaluating the efficacy of a continuous infusion of high-dose folinic acid plus FUra (given as an iv bolus) against FUra alone in the treatment of disseminated colorectal cancer.

MATERIALS AND METHODS

Eligibility.—Fifty patients with biopsy-proven, recurrent adenocarcinoma of the colon or rectum with no history of prior chemotherapy have been entered on this investigation. Because of a concurrent institutional trial of regional chemotherapy for patients with colorectal carcinoma metastatic to liver, all patients treated on this study were required to have metastatic disease outside the liver (with or without hepatic metastases) to be eligible. All patients had bidimensionally measurable disease, a performance status $\geq 50\%$ (Karnofsky scale), life expectancy ≥ 2 months, an absolute granulocyte count $\geq 2{,}000/\mu l$ and platelet count $\geq 150{,}000/\mu l$, total bilirubin ≤ 3.0 mg/dl, serum creatinine ≤ 3.0 mg/dl, and no history of prior cancer except basal cell carcinoma of the skin. Patients having received prior radiation therapy were eligible provided that radiotherapy had been completed at least 4 weeks before entering the study; however, patients with metastasis to the central nervous system were not eligible.

Pretreatment evaluation and follow-up studies.—Prior to the start of therapy all patients provided a complete history and underwent a physical examination; laboratory examination included a complete blood count with differential, platelet count, 18-function biochemical profile, chest x-ray, ECG, and abdominal and liver CT examination. Complete blood counts with differential and platelet counts were performed weekly; serum electrolytes, BUN, creatinine and liver function tests monthly. Tumor measurements were required monthly if easily accessible by physical examination or chest x-ray; otherwise every 2 months (or 2 courses of therapy) if liver CT was used for assessment of measurable disease.

Study design.—This study was reviewed and approved by the Institutional Review Board of the City of Hope National Medical Center. After determining eligibility but before entering into the study, all patients gave their written informed consent. The study utilized a partial crossover design in which patients with disease progression on the FUra arm were permitted to receive FUra plus folinic acid at the discretion of the patient's attending physician. Patients were stratified according to performance status (Karnofsky scale $\leq 70\%$ vs. $\geq 80\%$), prior radiotherapy (yes vs. no), and presence of hepatic metastasis (yes vs. no). All patients were then randomly assigned to receive either FUra or FUra plus high-dose folinic acid. Randomization was carried out using a complete block design within the various strata. The randomized assignments were made in

---

[1] Supported in part by Public Health Service Grant CA-33572, the Leukemia Society of America, and by a grant from Lederle Laboratories.

[2] Department of Medical Oncology and Therapeutics Research, Department of Biostatistics, and Division of Pediatrics, City of Hope National Medical Center, Duarte, CA.

[3] We thank the clinical nurse specialists and data managers who contributed to this study and Ms. Sunny Aure for secretarial assistance.

* *Reprint requests to:* James H. Doroshow, M.D., Dept. of Medical Oncology and Therapeutics Research, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010.

TABLE 1.—Characteristics of 50 patients randomized to treatment with FUra or FUra and folinic acid

Characteristic	FUra	FUra and folinic acid
No. of patients entered	27	23
Colon	20	15
Rectum	7	8
Male:female	10:17	9:14
Prior radiotherapy	6	6
Karnofsky performance status		
80–100%	22	19
$<80\%$	5	4
Mean age, yr	57	60
Range	37–77	38–82
Sites of disease		
Liver	19	15
Abdomen[a]	21	13
Lung	9	13
Soft tissue[b]	3	1
Bone	0	0

[a] Includes all extrahepatic structures, pelvis, and retroperitoneum.

[b] Includes lymph nodes and skin.

the Department of Biostatistics, City of Hope National Medical Center.

Treatment.—The therapy consisted of a 6-day infusion of 500 mg/m^2/day of folinic acid (calcium leucovorin; Lederle Laboratories, Pearl River, NY) initiated 24 hours prior to a 5-day course of FUra given by iv bolus at a dose of 370 mg/m^2/day; or FUra alone in the same dose, route, and schedule. Treatment was repeated every 28 days. Patients on both arms previously exposed to radiotherapy were initially treated with FUra at a dose of 250 mg/m^2/day (level 1); subsequent doses of FUra in these patients were increased to level 2 (300 mg/m^2/day), then level 3 (370 mg/m^2/day), where possible, based on hematologic and/or gastrointestinal toxicity. All drug dosages were calculated from surface area determinations based on ideal body weight. The folinic acid dose remained unchanged throughout the study; however, if more than moderate hematologic or gastrointestinal toxicity was observed on any course of therapy, the FUra dose was lowered one level. All patients received a minimum of 2 courses of therapy unless evidence of disease progression was documented after the first course of treatment and was associated with at least moderate hematologic or gastrointestinal toxicity.

Criteria for response.—Partial response was defined as a decrease of $\geq 50\%$ in the area (sum of the products of the diameters) of the measurable lesion(s), without evidence of new lesions for two consecutive evaluations separated by at least 1 month. The same criteria were used whether single or multiple lesions were evaluated. Disease progression was defined as an increase of $\geq 25\%$ in the area of the measurble lesion(s) or the appearance of new lesions. Patients with tumors not meeting these criteria for response or progression were considered stable. Response duration was measured from the onset of response until disease progression; duration of stable disease was calculated from the first day of therapy until progression. Survival was determined from the first day of treatment until death. All patient evaluations were performed by at least two of the authors.

Pharmacokinetics.—To determine the pharmacokinetics of high-dose, continuous-infusion folinic acid, blood samples were removed through an indwelling catheter from the arm contralateral to that into which the folinic acid was infused; 5-ml samples were removed into an iced, heparinized container immediately prior to and then 10, 20, and 30 minutes and 2, 6, 48, 72, 96, 120, and 132 hours after the folinic acid was begun. Samples were also obtained 10, 20, 30, 120, and 360 minutes after the completion of the folinic acid infusion. Plasma levels of D,L-folinate, L-folinate, D-folinate, 5-methyltetrahydrofolic acid, and total bioactive folates were measured by high-pressure liquid chromatography combined with a modification of standard bacterial growth assays for *Lactobacillus casei* and *Pediococcus cerevisiae* as recently described (*10*).

Statistical methods.—Patient characteristics were compared using either *t*-tests or chi-square tests depending on the nature of the data. Survival (time-to-event) curves were computed using the method of Kaplan and Meier (*11*), and the curves were compared using the log-rank test (*12*). Comparisons of toxicity between the treatments were made with the chi-square test.

RESULTS

Patient Characteristics

A total of 50 patients have been randomized on this trial. The median follow-up has been 10.1 months. The characteristics of the study population are presented in table 1. There are no significant differences between any features of the two treatment groups. It is important to note, however, that 2 patients randomized to receive FUra plus folinic acid were subsequently found to be ineligible for the study; 1 patient had primary pancreatic cancer at autopsy and 1 patient had a colonic primary that was found to be a rare mixed tumor consisting of both carcinoid and adenocarcinomatous elements on review of the original surgical specimen.

Response Rates and Durations

Twenty-seven patients who received FUra and 21 patients treated with FUra and folinic acid were evaluable for response and survival (table 2). No complete responses have been observed on this study; 1 patient treated with FUra and folinic acid who appeared to have complete disappearance of hepatic metastases on CT examination had a partial response confirmed by surgical re-exploration. Five (18%) of the 27 evaluable patients treated with FUra and 10 (48%) of 21 patients who received FUra and folinic acid

TABLE 2.—Therapeutic responses

	FUra	FUra and folinic acid
No. of evaluable patients	27	21[a]
Partial responses (%)	5 (18%)	10 (48%)
Duration, months	0.9+, 2.8, 6.4, 6.9, 7.4	2.2, 2.2, 3.5, 3.7, 3.8, 4.1+, 5.0, 5.5+, 6.4, 12.3
Stable disease	11	7
Progression	11	3
Too early	0	1

[a] One patient found to have primary pancreatic cancer at autopsy; 1 patient found to have a mixed carcinoid and adenocarcinoma of the colon on review of the primary surgical specimen.

Table 3.—Hematologic toxicity of patients treated with 370 mg/m^2/day of FUra

	FUra	FUra and folinic acid
	No. of patients	
No. of patients treated at level 3	23	18
WBC nadir × 10^3, cells/mm^3		
≥4.5	11	4
3.0–4.4	7	8
2.0–2.9	3	6
1.0–1.9	2	—
Platelet nadir × 10^3, cells/mm^3		
≥130	21	15
90–129	1	3
50–89	1	—

achieved partial responses, $P=0.02$. Duration of response, however, was not significantly different between the 2 treatment arms. The median time to disease progression was 3.9 months for the FUra arm and 8.0 months for FUra plus folinic acid, $P=0.006$. To date, 15 patients have been crossed over from FUra to FUra plus folinic acid; there have been no objective partial responses in this group. Median survival for patients randomized to receive FUra initially is 11.9 months (11 of 27 patients alive) versus 14.5 months for patients treated with FUra plus folinic acid (10 of 21 alive; $P=0.2$, log-rank test).

Toxicity

The drug-related toxicities noted in this study were usually transient, and when severe, easily controllable by reducing the dose of FUra to the next lower level. Severe leukopenia (WBC<2,000) was seen in only 2 of 41 patients treated at a dose of 370 mg/m^2/day (table 3). Moderate or severe diarrhea was found in approximately 25% of patients receiving this dose level (table 4). Significantly more patients treated with FUra and folinic acid developed moderate or severe stomatitis compared to patients receiving FUra alone ($P<0.02$, table 4). This was the only difference in toxicity found between the two treatment regimens. Other side effects of chemotherapy, including anemia, nausea and vomiting, erythematous skin changes, and hair loss, were mild and infrequent in both treatment programs. There were no treatment-related deaths.

Table 4.—Other toxicities of patients treated with 370 mg/m^2/day of FUra

	FUra	FUra and folinic acid
	No. of patients	
No. of patients treated at level 3	23	18
Diarrhea		
None	16	12
Mild (no therapy required)	2	1
Moderate (controlled by therapy)	4	3
Severe (iv fluids required)	1	2
Stomatitis[a]		
None	18	6
Mild (erythema, able to eat)	3	4
Moderate (mucositis; ulcers; able to eat)	2	5
Severe (mucositis; unable to eat)	0	3

[a] Degree of stomatitis significantly different between the 2 treatment groups, $P<0.02$.

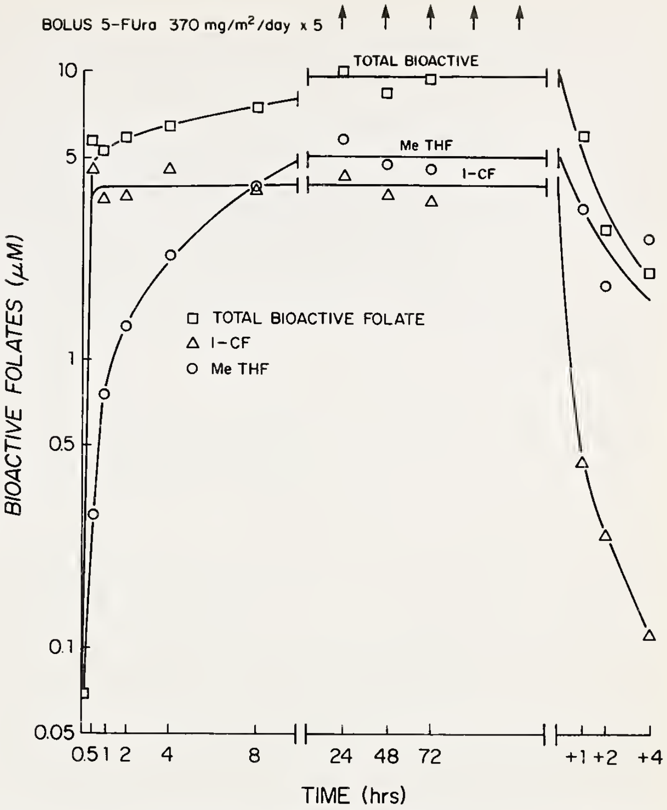


Figure 1.—Pharmacokinetic and metabolic profile of a representative patient treated with 500 mg/m^2/day of calcium leucovorin for 5 days as a continuous iv infusion. l-CF=L-citrovorum factor; MeTHF=5-methyltetrahydrofolate. Each arrow indicates a single dose of FUra.

Pharmacokinetics

The pharmacokinetic profile of a representative patient treated with a continuous infusion of folinic acid is shown in figure 1. While the L-folinic acid concentration increased rapidly after initiation of the D,L-folinate infusion, steady state levels of total biologically active folates were not reached for approximately 8 hours. As in this subject, the total biologically active level of plasma folates in most patients after an infusion of 500 mg/m^2/day of calcium leucovorin was approximately 10 *μM;* approximately half L-folinate and half 5-methyltetrahydrofolate. As expected, the decay of biologically active folates was prolonged by the 5-methyltetrahydrofolate species.

DISCUSSION

Over the past 10 years there have been numerous attempts to increase the therapeutic utility of FUra (*1-4*). Most of these efforts have resulted in encouraging pilot and phase II trials that, unfortunately, have not been confirmed by prospective, randomized comparisons of the drug combination against FUra alone. Recent preclinical studies from several institutions (*5-7*), however, have strongly suggested that some human colorectal cancers are resistant to the cytotoxic effects of FUra because they lack a sufficient level of intracellular reduced folates to promote maximal binding of 5-fluoro-2′-deoxyuridine monophosphate to thymidylate synthase. In support of the clinical application of this finding, Machover et al. (*8*) reported that 9 of 16 patients with previously untreated metastatic colorectal cancer achieved an objective response with the combination of FUra (370–400 mg/m^2/day) and folinic acid (200 mg/m^2/day by iv bolus injection immediately prior to FUra).

Straw and colleagues (*13*) have demonstrated that the biologically active folates L-folinate and 5-methyltetrahydrofolate disappear rapidly from plasma after iv bolus administration. Furthermore, a detailed evaluation of the concentration-effect curves for FUra and D,L-folinic acid against human cell lines has revealed that concentrations of $\geq 1\ \mu M$ biologically active reduced folates promote maximal enhancement of cytotoxicity (Keyomarsi K, and Moran RG, submitted for publication). Thus, in this study we chose to administer D,L-folinate by a constant iv infusion; our steady state target level of bioactive folates was 1 to 10 μM. As shown in figure 1, and in a group of 12 patients studied to date (data not given), this target figure was easily achieved with a folinic acid dose of 500 mg/m^2/day.

The preliminary evaluation of our ongoing City of Hope trial suggests that both response rate and time to progression are significantly improved by the addition of folinic acid to FUra. It is noteworthy that this was achieved despite the exclusion of patients with liver as a solitary site of metastasis from the study because of a concurrent institutional trial. The major test for this investigation, however, is whether the addition of folinic acid to FUra produces a significant prolongation of survival for patients with advanced colorectal cancer compared to FUra when used as a single agent. At this early stage in the trial (median follow-up of 10.1 months), there is no obvious survival benefit for either arm of the study. However, the median survivals for both arms (11.9 and 14.5 months) are in general longer than is usually reported for either phase II (*14*) or phase III (*1*) evaluations of patients with metastatic colorectal cancer. Although there have been no objective responses to date in the group of patients treated with FUra and folinic acid after their disease progressed on FUra, we cannot exclude a beneficial effect on the survival of the FUra arm produced by subsequent FUra and folinic acid therapy. Our evaluation of the toxicities associated with this trial revealed a moderate level of leukopenia and diarrhea on both arms of the study. Stomatitis was the only drug-induced side effect that was increased by the coadministration of folinic acid.

In summary, an interim analysis of this ongoing study suggests that a continuous infusion of folinic acid may enhance the efficacy of FUra in the treatment of advanced colorectal cancer. However, because of the high cost of folinic acid in the doses used for our regimen, we reserve recommending this mode of treatment until definitive survival data from our trial matures.

REFERENCES

(*1*) Buroker TR, Moertel CG, Fleming TR, et al: A controlled evaluation of recent approaches to biochemical modulation or enhancement of 5-fluorouracil therapy in colorectal carcinoma. J Clin Oncol 3:1624–1631, 1985.

(*2*) Kemeny NE, Ahmed T, Michaelson RA, et al: Activity of sequential low-dose methotrexate and fluorouracil in advanced colorectal carcinoma: Attempt at correlation with tissue and blood levels of phosphoribosylpyrophosphate. J Clin Oncol 2:311–315, 1984.

(*3*) Woodcock TM, Martin DS, Damin LAM, et al: Combination clinical trials with thymidine and fluorouracil. Cancer 45:1135–1143, 1980.

(*4*) Meshad MW, Evin TJ, Kufe D, et al: Phase I trial of combination chemotherapy with PALA and 5-FU. Cancer Treat Rep 65:331–334, 1981.

(*5*) Houghton JA, Maroda SJ, Phillips JO, et al: Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinomas in mice. Cancer Res 41:144–149, 1981.

(*6*) Evans RM, Laskin JD, Hakala MT: Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. Cancer Res 41:3288–3295, 1981.

(*7*) Yin MB, Zakrzewski SF, Hakala MT: Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. Mol Pharmacol 23:190–197, 1983.

(*8*) Machover D, Schwarzenberg L, Goldschmidt E, et al: Treatment of advanced colorectal and gastric adenocarcinoma with 5-FU combined with high-dose folinic acid: A pilot study. Cancer Treat Rep 66:1803–1807, 1982.

(*9*) Madajewicz S, Petrelli V, Rustum YM, et al: Phase I–II trial of high dose calcium leucovorin and 5-fluorouracil in advanced colorectal cancer. Cancer Res 44:4667–4669, 1984.

(*10*) Newman EM, Tsai JF: Microbiological analysis of 5-formyltetrahydrofolic acid and other folates using an automatic 96-well plate reader. Anal Biochem. In press.

(*11*) Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481, 1958.

(*12*) Peto R, Pike MC, Armitage P, et al: Design and analyses of randomized clinical trials requiring prolonged observations of each patient: Part 2, analysis and examples. Br J Cancer 34:1–39, 1977.

(*13*) Straw JA, Szapary D, Wynn WT: Pharmacokinetics of the diastereoisomers of leucovorin after intravenous and oral administration to normal subjects. Cancer Res 44:3114–3119, 1984.

(*14*) Kemeny N, Yagoda A, Braun D, et al: Therapy for metastatic colorectal carcinoma with a combination of methyl-CCNU, 5-fluorouracil, vincristine and streptozotocin (MOF-Strep). Cancer 45:876–881, 1980.

A Northern California Oncology Group Randomized Trial of Leucovorin Plus 5-Fluorouracil Versus Sequential Methotrexate, 5-Fluorouracil, and Leucovorin in Patients With Advanced Colorectal Cancer Who Failed Treatment With 5-Fluorouracil or 5-Fluorodeoxyuridine Alone[1]

Frank H. Valone,[2,*] Marsha Kohler,[3] Karen Fisher,[3] John Hannigan,[3] Marshall Flam,[4] David Gandara,[5] Charles Hendrickson,[6] Eli Richman,[7] and Kou-Ping Yu[8]

ABSTRACT—**The current study was initiated to confirm preliminary reports that 20% or more of patients with colorectal cancer who fail treatment with 5-fluorouracil (FUra) will respond to treatment with either leucovorin plus FUra or with sequential methotrexate, FUra, leucovorin. One hundred two patients with advanced, measurable colorectal cancer who failed treatment with FUra and/or 5-fluorodeoxyuridine (FUdR) were randomized to treatment with either high-dose leucovorin plus FUra (Arm B) or sequential methotrexate, FUra, leucovorin (Arm C). In this interim report, 92 patients were evaluable for toxicity and 89 patients were evaluable for response. Grade 3 or 4 nonhematologic toxicity which was primarily gastrointestinal was experienced by 25% of patients on both treatment arms during at least 1 treatment cycle. Hematologic toxicity was minimal. Among 43 evaluable patients on Arm B, there were 2 complete responses (5%) and 1 minor response (3%). Among 46 evaluable patients on Arm C, there was 1 complete response (2%), 1 partial response (2%), and 6 minor responses (14%). The median time to treatment failure was 2.2 months on Arm B and 3.5 months on Arm C. The median survival was 8.3 months on Arm B and 8.7 months on Arm C. Colorectal cancers that are resistant to FUra are cross-resistant to both experimental combinations.—NCI Monogr 5:175–177, 1987.**

Numerous studies have demonstrated that sequential methotrexate, FUra, leucovorin (*1–5*), or leucovorin plus FUra (*6–8*) are effective treatments for patients with advanced colorectal cancer. Both treatment regimens have objective response rates of greater than 30%, which is substantially greater than would be expected for treatment with FUra alone. In addition, these two regimens may have a salvage rate which is clinically significant, albeit small, for patients who failed prior treatment with FUra. The Northern California Oncology Group (NCOG) has initiated a 2-part study of patients with advanced, measurable colorectal carcinoma which is designed to confirm these 2 observations in a prospective, randomized manner. A 3-arm trial randomizes previously untreated patients to treatment with either FUra alone (Arm A), leucovorin plus FUra (Arm B), or sequential methotrexate, FUra, and leucovorin (Arm C). A separate 2-arm trial examines cross-resistance between FUra and the 2 experimental combinations. Patients who failed prior FUra (or FUdR) either on or off the 3-arm trial are randomized to treatment with either Arm B or Arm C. This report presents the results of the 2-arm trial.

METHODS

Patient selection.—Patients with measurable locally recurrent or metastatic cancer of the colon or rectum who failed treatment with FUra and/or FUdR were eligible for entry in the study. Additional eligibility criteria were expected survival greater than 60 days, Karnofsky performance status >40% and adequate hepatic function (bilirubin <7 mg/dl, SGOT <4 times normal), adequate renal function (creatinine <1.5 mg/dl or creatinine clearance >50 ml/min) and adequate hematologic parameters (WBC >4,000/mm^3, platelets >100,000/mm^3). Patients were stratified by Karnofsky performance status, history of prior radiotherapy, treatment with FUra or FUdR, and response to prior chemotherapy.

Treatment.—Eligible patients were randomized to treatment with 1 of 2 combination chemotherapy regimens. Arm B was the regimen described by Machover et al. (*6*): leucovorin (200 mg/m^2) by iv push followed immediately by FUra (400 mg/m^2) iv over 15 minutes. Both drugs were given on days 1 through 5 of a 28-day cycle. The dosage of FUra was increased by 10% if no systemic toxicity occurred during the preceding treatment cycle and if a CBC on day 15 revealed WBC >3,000/mm^3 and platelets >100,000/mm^3. Arm C was sequential methotrexate, FUra, and leucovorin. Treatment started with methotrexate (50 mg/m^2) orally every 6 hours for 5 doses. FUra (500 mg/m^2) was given by iv bolus 24 hours after starting methotrexate (immediately after the last methotrexate dose). Leucovorin (10 mg/m^2) was then given orally every 6 hours for 6 doses

---

ABBREVIATIONS: CR = complete remission; FUdR = 5-fluorodeoxyuridine; MR = minor response; NCOG = Northern California Oncology Group; PR = partial response; SD = stable disease.

[1] Supported in part by Public Health Service Grants CA-21744 and CA-39098.

[2] Veterans Administration Medical Center, San Francisco, CA.
[3] Northern California Oncology Group Headquarters, Palo Alto, CA.
[4] San Joaquin Valley CCOP, Fresno, CA.
[5] Veterans Administration Medical Center, Martinez, CA.
[6] Kaiser Foundation Hospital, San Francisco, CA.
[7] East Bay Oncology Group, Oakland, CA.
[8] Kaiser Foundation Hospital, Santa Clara, CA.
* *Reprint requests to:* Frank H. Valone, M.D., Veterans Administration Medical Center (111K), 4150 Clement Street, San Francisco, CA 94121.

TABLE 1.—Toxicity of treatment with leucovorin plus FUra (Arm B) or sequential methotrexate, FUra, leucovorin (Arm C) in patients with colorectal cancer previously treated with FUra

	Toxicity grade	No. of patients	
		Arm B	Arm C
Total patients		49	53
Evaluable patients		43	46
Hematologic toxicity	3	0	2
	4	2	1
	5	0	0
Nonhematologic toxicity	3	8	11
	4	1	1
	5	0	0

starting 6 hours after FUra. This was a modification of previously described regimens (*3,9*) and was based on studies of optimal sequencing of these drugs in vitro (*10,11*). Optimal synergy between methotrexate and FUra requires micromolar concentrations of methotrexate. Thus, methotrexate levels were measured immediately before giving FUra in the first 2 cycles. The dosage of methotrexate was increased to 70 mg/m^2 for those patients with methotrexate levels below 1 μM and the levels were remeasured. Treatment on Arm C was initially given weekly but, because of severe unpredictable gastrointestinal toxicity, the scheduling was changed to every other week after the study was open for 6 months. Lederle Laboratories (Pearl River, NY) provided the methotrexate and the leucovorin used in this study.

Evaluation of response to treatment.—All patients who received treatment were evaluable for toxicity. Toxicity was graded using standard NCOG criteria (*3*). Patients were evaluable for response after completion of 4 weeks of treatment. Response to treatment was evaluated every 8 weeks. Response criteria for each site of disease were defined as follows: Complete response (CR)—complete disappearance of all detectable disease for 1 month or more; partial response (PR)—greater than 50% decrease in the product of the longest perpendicular diameters of a clearly measurable lesion for 1 month or more with no increase in other lesions or new areas of disease; minor response (MR)—between 25% and 50% tumor reduction as defined for PR; stable disease (SD)—less than 25% increase in any measurable lesion and no new areas of malignant disease. A patient's overall response to treatment was scored as the most severe disease activity at any site. Time to progression and survival were measured from the date of randomization.

RESULTS

One hundred two patients were entered into the study (49 on arm B and 53 on Arm C). The patient characteristics were equally distributed on both arms except in the number of patients who received prior radiotherapy (13 on Arm B and 7 on Arm C). The mean age was 61.7 years (range:38 to 89), 57% were males, and the mean Karnofsky performance status was 85%. The patients' previous treatments were FUra (87%), FUdR (8%), and FUra and FUdR (5%). The median times from beginning FUra and/or FUdR to study entry were [illegible] months on Arm B and 6.5 months on Arm C. Forty-one percent of the patients reportedly responded to prior chemotherapy. However, the objective criteria described in the Methods section were not used to evaluate the patients' responses to prior treatment. For patients on Arm C, 79% achieved methotrexate levels (drawn immediately before FUra) greater than 1 μM which is the minimal level required for synergy in vitro. The average methotrexate level was 2 μM.

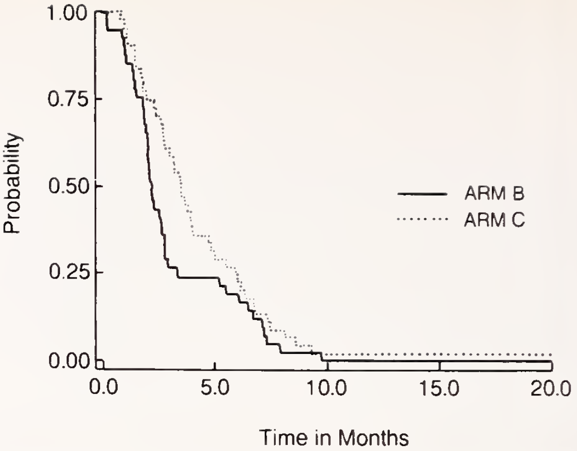


FIGURE 1.—Kaplan-Meier curve of the time to treatment failure for all evaluable patients as of June 1986. Forty-one patients on Arm B and 44 patients on Arm C have failed.

This study has become mature, so 43 patients on Arm B (87%) and 46 patients on Arm C (87%) were evaluable for response (table 1). Of the remaining patients, 5 on Arm B and 4 on Arm C were too early to evaluate, 1 on Arm B and 2 on Arm C were not evaluable, and 1 on Arm C was not eligible. The overall response to treatment was poor. On Arm B there were 2 CR (5%) and 1 MR (3%). On Arm C there were 1 CR (2%), 1 PR (2%), and 6 MR (14%). The median times to treatment failure (fig. 1) were 2.2 months on Arm B and 3.5 months on Arm C ($P=0.075$ by the log-rank test). The median survivals (fig. 2) were 8.3 months on Arm B and 8.7 months on Arm C ($P=0.35$ by the log-rank test).

Toxicity on both treatment arms was mild. Table 1 gives the maximal degree of toxicity experienced by each patient

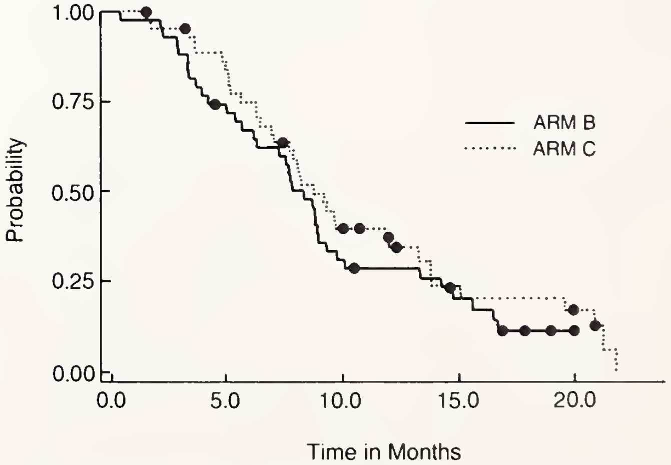


FIGURE 2.—Kaplan-Meier curve of patient survival for all evaluable patients as of June 1986. Thirty-six patients on each arm have died.

during any treatment cycle. Two patients on arm B and three patients on Arm C had NCOG grade 3 or 4 hematologic toxicity. Most patients (42 on Arm B and 44 on Arm C) had NCOG grade 1 or 2 nonhematologic toxicity, predominantly mucositis or diarrhea. Nine patients on Arm B and 12 patients on Arm C had grade 3 or 4 nonhematologic toxicity. Most of the cases on Arm C with this degree of toxicity were receiving weekly treatment, which was the schedule at the start of the study. No patient died from treatment-related causes.

DISCUSSION

The current study indicates that patients with colorectal cancer who fail treatment with FUra and/or FUdR are cross-resistant to the 2 FUra combination treatments: high-dose leucovorin plus FUra (Arm B), and sequential methotrexate, FUra, and leucovorin (Arm C). Less than 10% of the patients treated with either combination had an overall major (CR plus PR) response to treatment. The failure to confirm the higher response rates reported previously was probably not due to inadequate drug dosage or scheduling, since both treatment arms were based on active regimens and over 80% of the patients experienced some toxicity during treatment. The patients in this study appeared to be ideal for detecting a significant response rate to treatment. They had a mean Karnofsky performance status of 85% and had relatively slowly progressive disease as shown by the median 6- to 7-month interval between initiating FUra and/or FUdR and entry onto this study. Moreover, 41% of the patients reportedly responded to prior chemotherapy. Thus, patient selection and small patient numbers probably account for the observation in prior phase II studies that leucovorin plus FUra and sequential methotrexate, FUra, and leucovorin can salvage some patients with colorectal cancer who failed treatment with FUra.

This 2-arm study was part of a larger study in which untreated patients were randomized to FUra alone (Arm A) or to leucovorin plus FUra or to sequential methotrexate, FUra, and leucovorin. Through June 30, 1986, 216 patients have been entered into this 3-arm trial. Further accrual is necessary, however, to determine if either combination treatment is superior to FUra alone in terms of response and survival for untreated patients.

REFERENCES

(*1*) Weinerman B, Schacter B, Schipper H, et al: Sequential methotrexate and 5-FU in the treatment of colorectal cancer. Cancer Treat Rep 66:1553–1555, 1982.

(*2*) Hermann R, Spehn J, Beyer JH, et al: Sequential methotrexate and 5-fluorouracil: Improved response rate in metastatic colorectal cancer. J Clin Oncol 2:591–594, 1984.

(*3*) Ignoffo RJ, Friedman MA, Gribble M, et al: Phase II study of sequential methotrexate and 5-FU plus mitomycin and leucovorin in patients with disseminated large bowel cancer: A Northern California Oncology Group Study. Cancer Treat Rep 68:983–988, 1984.

(*4*) Kemeny NE, Ahmed T, Michaelson RA, et al: Activity of sequential low-dose methotrexate and fluorouracil in advanced colorectal carcinoma: Attempt at correlation with tissue and blood levels of phosphoribosylpyrophosphate. J Clin Oncol 2:311–315, 1984.

(*5*) Leone BA, Romero A, Rabinovich MG, et al: Sequential therapy with methotrexate and 5-fluorouracil in the treatment of advanced colorectal carcinoma. J Clin Oncol 4:23–27, 1986.

(*6*) Machover D, Goldschmit E, Chollet P, et al: Treatment of advanced colorectal and gastric adenocarcinomas with 5-fluorouracil and high-dose folinic acid. J Clin Oncol 4:685–698, 1986.

(*7*) Bryne P, Smith F, Treat MW, et al: 5-Fluorouracil and higher dose folinic acid treatment of colorectal carcinoma patients. Proc ASCO 2:121, 1983.

(*8*) Bertrand M, Doroshow JH, Multhauf P, et al: High-dose continuous infusion folinic acid and bolus 5-fluorouracil in patients with advanced colorectal cancer: A phase II study. J Clin Oncol 4:1058–1061, 1986.

(*9*) Benz C, Silverberg M, Cadman E: Use of high-dose oral methotrexate sequenced at 24 hours with 5-FU: A clinical toxicity study. Cancer Treat Rep 67:297–299, 1983.

(*10*) Benz C, Tillis T, Tattelman E, et al: Optimal scheduling of methotrexate and 5-fluorouracil in human breast cancer. Cancer Res 42:2081–2086, 1982.

(*11*) Benz C, Cadman E: Modulation of 5-fluorouracil metabolism and cytotoxicity by antimetabolite pretreatment in human colorectal adenocarcinoma, HCT-8. Cancer Res 41:994–999, 1981.

Comparison of Unique Leucovorin and 5-Fluorouracil "Escalating" and "Maximum" Dosage Strategies[1]

Howard W. Bruckner,[2,*] Nicholas J. Petrelli,[3] Donald Stablein,[4] Joel Novak,[4] and Robert Mayer[5] for the Gastrointestinal Tumor Study Group

ABSTRACT—**In this study (GI 6384), regimens are compared which, in phase II investigations, produced 2-fold increases in the historical rates of objective and minor responses. These regimens were associated with overall survival exceeding 1 year. In arm 1, 5-fluorouracil (FUra) alone, 500 mg/m^2 on days 1 through 5 was escalated in 25-mg/m^2 increments in monthly courses to produce mild to moderate toxicity. This allows an examination of dose-response relationships and comparisons of therapeutic index. In arm 2, leucovorin (LV), was escalated from 25 to 250 to 500 mg/m^2 beginning 1 hour before a bolus FUra (30 mg/kg) every 3 weeks. Arm 3 (not previously tested) employed LV (25 mg/m^2) 1 hour before FUra (600 mg/m^2) given weekly for 6 weeks. It tested the efficacy of low-dose LV. Arm 4 tested high-dose LV (500 mg/m^2) as a 2-hour infusion beginning 1 hour before a bolus FUra (600 mg/m^2) weekly for 6 weeks. In a preliminary analysis of this study, findings are statistically consistent with the anticipated high frequencies of objective response. It also finds evidence of biological activity across the wide range of LV dosages and that LV produces an apparent favorable change in FUra side effects from hematological to gastrointestinal toxicities. One or more regimens may favorably change the anticipated prognosis of patients with measurable cancer of the colon and rectum.**—NCI Monogr 5:179–184, 1987.

The Gastrointestinal Tumor Study Group (GITSG) project GI 6384 is a controlled prospective clinical trial examining several strategies proposed as the optimum method for using FUra to treat patients with advanced measurable carcinoma of the colon and rectum. In pilot studies, these regimens produced 2-fold increases in the historical rates of response and overall survival. These regimens are not under investigation by others and offer several possible advantages including simplicity of schedule and relative absence of limiting bone marrow toxicity compared to alternative methods of producing biochemical modulation of FUra.

Arm I is a refinement of the Central Oncology Group (*1*) and Dana-Farber (*2,3*) loading schedules. This schedule provided an opportunity to determine, by direct comparisons, whether biochemical modulation with LV can improve either the results or therapeutic index of treatment with FUra. Arm 1 is the most dosage-intensive FUra control in current trials. The Central Oncology Group loading regimen produced a 33% rate of response ($P = .002$) and a median survival of 56 weeks ($P = .082$) compared to three standard less intensive FUra regimens (*1*). The Dana-Farber regimen produced a 38% rate of response, (12% nonstandard), a 14-month median survival and, for all responders, a 28-month median survival (*3*). Investigators suspected that optimum benefit often required some clinical manifestation of side effects (*1–3*). The current loading regimen attempts both to achieve the moderate level of toxicity associated with therapeutic benefit in earlier studies and to avoid the high incidence of severe toxicity found in earlier studies (less than 500 granulocytes per mm^3 in 61%, severe stomatitis in 38%, fever in 12%, hospitalization in 11%, neurological side effects in 8%, and documented infection in 4%) by use of an initial starting dosage of 500 mg/m^2 rather than the 575-mg/m^2 dosage of FUra. With dose escalation, this regimen provides the [illegible] control test to determine whether biochemical modulation only duplicated FUra dosage intensification or truly improves response and survival due to treatment. LV must be proven to be, as investigators believe, more than an expensive method of escalating the dosage of FUra.

Arm II combines an escalating LV regimen with intermittent FUra treatment (*4–7*). This intermittent regimen may offer 25% to 50% savings in drugs and time. Each patient was treated with a minimum effective dosage of LV. The drug dosage was escalated after 3 treatments at each dosage level (9 weeks), if an objective response was absent. FUra can sometimes be effective alone or when modulated by low or intermediate concentrations of reduced folates (*4–15*). In the initial clinical trials of LV as a potentiator of FUra, an intermittent low-dose schedule was selected for trial in order to avoid toxicities. As little as 15 mg/m^2 of LV in conjunction with FUra (either 30 mg/kg/day as an infusion on days 1 to 4 or as 12- to 15-mg/kg bolus injections on days 1 and 2) sometimes produced severe leukopenia and thrombocytopenia and sometimes produced responses after FUra alone had failed (*4*). The intermittent LV regimen produced a 19% rate of standard clinical response. An additional 29% of patients had laboratory and x-ray evidence of tumor regression (*5–7*). Median survival was 15 months. Responders, including minor responders, did bet-

---

ABBREVIATIONS: CH_2FH_4 = 5,10-methylenetetrahydrofolate; GITSG = Gastrointestinal Tumor Study Group; FdUMP = fluorodeoxyuridylate; LV = leucovorin; TS = thymidylate synthetase.

[1] Supported by grant number CA-34182 from the National Institutes of Health, Bethesda, MD.

[2] Department of Medicine and Neoplastic Diseases, Mount Sinai School of Medicine, New York, NY.

[3] Department of Surgery, Roswell Park Memorial Institute, Buffalo, NY.

[4] The EMMES Corporation, Potomac, MD.

[5] Dana-Farber Cancer Institute, Boston, MA.

[*] *Reprint requests to:* Howard W. Bruckner, M.D., 1 Gustave L. Levy Place, New York, NY 10029.

ter than patients with progressive disease. In addition, patients previously treated with FUra demonstrated objective evidence of benefit (*5–7*). Two patients appeared to respond to increasing doses of LV if their initial dosage was ineffective. One patient responded to an increase from 25 mg to 100 mg and another from 25 mg to 200 mg.

Arm III tested the efficacy of low-dose LV (25 mg/m^2) in conjunction with a convenient standard weekly FUra schedule. This specific low-dose LV regimen has not previously been evaluated. It provides a new LV-FUra schedule identical to that in the fourth arm, specifically for comparison to the fourth high-dose LV arm.

Testing of LV dosage is a major objective of this trial. A wide range of LV dosages have sometimes been observed to be effective in the laboratory and in phase I–II clinical trials (*10–18*) at a concentration of LV ranging from 5×10^{-7} *M* to 10^{-5} *M* (*11,15,18*). Fluorodeoxyuridylate (FdUMP) binds more tightly to thymidylate synthetase (TS) in the presence of the cofactor 5,10-methylenetetrahydrofolate (CH_2FH_4). TS and CH_2FH_4 form a covalent ternary complex which dissociates slowly (*10–13*). LV increases the cytotoxic potency of fluorinated pyrimidines (*10,11,18*). The minimum effective concentration of reduced folates which increases the rate of association and decreases the rate of dissociation of the enzyme complex in vitro differs substantially from cell line to cell line (*11,14,15,18*) and is limiting in these reactions (*12*). The concentration of LV may be quite low and can differ for enzymes even if they are derived from identical species and tissues, such as different murine carcinomas of the colon (*14,15*).

Reduced folates affect all tested TS enzymes, including those found in normal cells. Therefore, LV may alter the side effects of FUra. This was another consideration in deciding to test a high-dose as well as a low-dose LV regimen.

Arm IV tests a high dose of LV (500 mg/m^2) because only high concentrations of LV appear to produce biochemical modulation in some tumor systems (*16–19*). The 2-hour infusions are empiric in theory, intended to produce an efficient and prolonged exposure to LV. It produces brief serum levels of LV compared to the half-life of the enzyme complex (*19,20*). The high-dose LV regimen is noteworthy for both the exceptionally high response rate in patients who have received prior chemotherapy (36%) and the duration of the responses in refractory patients (median of 10 months) (*19,20*). A phase III study, testing a 6-day 500-mg/m^2/day infusion of LV with low-dose bolus FUra on days 2 to 6, provided further support for test of this high-dose regimen. It produced preliminary evidence that response rates were significantly superior, 6 of 15 (40%) vs. 4 of 14 (28%), to those of patients treated with bolus FUra alone (*21,22*).

Without LV, other single-institution and collaborative trials found half of these rates of response (10% to 14%) and median (6 to 7 months) survivals (*1,23*). In completed comparative trials without LV, neither response nor overall survival has ever been found to improve convincingly (*24*). LV regimens provide the first systemic treatments which may improve these dismal standard results.

METHODS

Patient selection.—In order to be eligible, patients had to have metastatic carcinoma of the colon or rectum that was unsuitable for curative treatment. One or more mass lesions due to tumor, other than the primary, had to be clearly measurable by physical examination, chest x-ray, CAT scan (lesion of 4 cm or greater); or scintogram scans (not gamma camera) if the defect was 5 cm or greater in diameter. Patients must have had a white blood cell count of 4,000/mm^3 or greater, a platelet count of 150,000/mm^3 or greater, and a hemoglobin of 10 g/dl with or without prior transfusion. Patients must have recovered from surgery and been at least 2 weeks postlaparotomy or 3 weeks postsurgery involving bowel resection or bypass. Patients with an elevated white blood cell count (12,000/mm^3 or greater), or fever (99° F or greater) must have had no evidence of infection, tested by chest x-ray, and urine and blood surveillance cultures. Eligibility required consent in accordance with institutional and the Department of Health and Human Services requirements.

Therapy was started within 5 days of randomization. Randomization was performed based on two stratification criteria: colon vs. rectum and entirely ambulatory vs. partially ambulatory.

Patients were ineligible if they were 50% or more bedridden, had severe nausea or vomiting, had a chronic nidus of infection, or were using any anti-inflammatory therapy. They were also ineligible if they had prior chemotherapy or radiotherapy or any second neoplasm other than basal cell carcinoma.

Toxicities were defined as follows, for purposes of dosage modification: grade 1 (mild)—stomatitis, mild ulcers, mild diarrhea, vomiting 2 to 3 times per day, a white blood cell count of 3,000 to 3,900/mm^3, or a platelet count of 100,000 to 149,000/mm^3; grade 2 (moderate)—stomatitis that required analgesic or topicals, 4 to 6 diarrheal bowel movements per day, a skin rash that required care, a white blood cell count of 2,000 to 2,900/mm^3 or a platelet count of 75,000 to 99,000/mm^3; grade 3 (severe)—stomatitis with bleeding or open ulcers, 7 to 10 bloody diarrheal bowel movements per day, an exfoliative rash, a white blood cell count of 1,000 to 1,900/mm^3 or a platelet count of 25,000 to 74,000/mm^3; and grade 4 (life-threatening)—worse than above.

Arm I.—The FUra loading schedule initially consisted of bolus injections of FUra (500 mg/m^2) given for 5 consecutive days, every 28 days. The complete blood count was checked before the fourth and fifth treatment to insure that the white blood cell and platelet counts were both greater than 4,000/mm^3 and 100,000/mm^3, respectively, and greater than 50% of the initial counts.

The dosage of FUra was increased 25 mg/m^2 on days 1 to 5 with each course, provided that there was no limiting toxicity, such as grade 1 gastrointestinal toxicity, other nonhematologic grade 2 toxicity, or a nadir granulocytopenia of less than 1,500/mm^3. FUra dosage was deescalated in 25-mg/m^2 increments per course, if there was severe nonhematologic or hematologic life-threatening toxicity, less than 500 granulocytes per mm^3, or infection associated with granulocytopenia.

Arm II.—Low-dose LV with LV escalation and high-dose FUra consisted of a single treatment once every 3 weeks. An infusion of LV preceded each bolus injection of FUra. FUra (1,000 mg/m^2) was administered as a bolus, not to exceed 30 mg/kg based on ideal weight, exactly 1 hour after starting the LV infusion. The rate of the bolus injection was 500 to 1,000 mg/minute, the maximum comfortable rate.

The second and third courses of treatment allowed FUra escalation in order to achieve use of the maximum safe dose of FUra. The goal was to achieve a nadir granulocyte count of near 1,000/mm^3, which allowed a safety margin for possible further side effects with future larger doses of LV. FUra escalation was performed in 100 mg/m^2 increments, only at the second and third courses, provided grade 2 toxicity was absent and the maximum dose of FUra was not greater than 1,200 mg/m^2 or 32.5 mg/kg based on ideal weight. The dosage increment of FUra was based on the observed steep dose–response curve for producing granulocytopenia (*7*).

The dosage of FUra was reduced in 10% increments, if the nadir granulocyte count was less than 500/mm^3 or grade 2 toxicity produced unacceptable symptoms. The dosage of FUra was reduced in 20% steps, if grade 3 or 4 toxicities produced unacceptable symptoms, or if granulocytopenia was associated with fever (with or without documented infection).

Granulocyte counts were required regardless of the total white blood count in order to perform dosage adjustments. Selective severe or life-threatening granulocytopenia may occur in the presence of a normal peripheral white blood cell count.

The initial dosage of LV was 25 mg/m^2. This was increased to 250 mg/m^2 and then to 500 mg/m^2 if there was no objective response after 3 treatments at each dose level. The 25-mg/m^2 dose of LV was administered in 100 ml of normal saline as a 10-minute iv infusion. Escalation of LV first began on day 64 provided: FUra had been escalated to 1,200 mg/m^2 or subsequently deescalated; there had been no immediately preceding moderate toxicity; the immediately preceding granulocyte nadir was greater than 1,000/mm^3; and the patient had stable disease without an objective response. The 250-mg/m^2 and 500-mg/m^2 doses of LV were administered as 1- and 2-hour infusions, respectively, in 200 ml of normal saline. FUra was delivered as a rapid bolus immediately at the end of the infusion of 250 mg/m^2 of LV and 1 hour after the start of the 500-mg/m^2 infusion of LV.

Arm III.—Treatment was given weekly for 6 weeks followed by a 2-week rest period. Only 25 mg/m^2 of LV was infused over 10 minutes beginning exactly 1 hour before the bolus 600-mg/m^2 injection of FUra. The dosage of LV was not adjusted. It was withheld when FUra was withheld.

The dosage of FUra was adjusted in 100-mg/m^2 increments. There was 1 dose level decrease if the white blood cell count was less than 4,000/mm^3 or the platelet count less than 100,000/mm^3, or if there was mild stomatitis, ulcers, or increased frequency of bowel movements (2 to 3 per day). Treatment was resumed at the former greater dosage once there was recovery. Both drugs were withheld if the white blood cell count was less than 3,000/mm^3 or the platelet count less than 50,000/mm^3, or if there was moderate dermatologic, oral, gastrointestinal, or diarrheal toxicity. Treatment was resumed once there was recovery; however, FUra was decreased 1 dosage level.

Arm IV.—Treatment was identical to the above low-dose treatment, except that LV (500 mg/m^2) was infused over 2 hours. Sixty minutes after the start of the LV infusion the FUra was administered as a 600-mg/m^2 bolus injection, at the maximum comfortable rate.

Nausea, vomiting, and gastroenteritis were treated symptomatically. If antiemetics were ineffective, the dosage of FUra, not LV, could be decreased in 10% increments.

Both history and physical examination sought possible evidence of cerebellar ataxia. Treatment was held until this was resolved.

Patients continued treatment until there was objective evidence of progression. Patients were followed after progression, both for survival analysis and in order to determine the impact of any subsequent treatment on survival.

Laboratory parameters include: complete blood count with platelets and differential every week; urinalysis; standard chemistries, every 3 to 5 weeks; sonogram or CAT scan; chest x-ray; and carcinoembryonic antigen titers at 8 then every 12 weeks, and at the time of any clinical indication of progression or major toxicity.

Responses were defined as follows: complete—absence of all clinically and radiographically detectable tumor; partial—reduction of at least 50% of the product of the longest perpendicular signal tumor diameters; stable disease—a less than 25% reduction or increase in the product of the longest perpendicular diameter of the indicator lesion or a less than 25% reduction or increase in the sum of the distances below both costal margins at the midclavicular and the xiphoid lines; progression—a greater than 25% increase in the product of the longest perpendicular diameters of any indicator lesion, or a 25% increase in the sum of the distances below the costal margin and xiphoid process, or appearance of new lesions. Decreases in tumor size not meeting partial response criteria, sometimes referred to as "minor responses," will be examined separately in order to determine their impact on survival. Such changes were frequently observed in some pilot studies (*3,7*).

A period of 2 months must have elapsed between the start of treatment and the date of first assessment of objective response other than progression of a measurable tumor. Partial and complete responses required at least a 30% reduction in the sum of liver measurement below the costal margin at the midclavicular line and at the xiphoid process. In order for tumor regression or stable disease to have been recorded, there must have been no increase in any other indicator lesion or appearance of a new lesion or significant deterioration in weight, symptoms, or performance status.

The study was designed with a projected sample size of 109 patients per arm. When complete, it will provide a 95% pairwise power to detect a 20% improvement in response rate using a 1-tailed 5% test. This assumes that standard therapy will produce a 15% rate of response. The study was designed to have a power excess of 80% to detect a 15% improvement in the rate of response. It also will provide a 90% pairwise power to detect a 50% improvement in survival using a 5% 1-tailed log-rank criterion.

An initial analysis was required after the accrual of 20 evaluable patients to each of the test regimens. An additional analysis will be performed every 6 months in order to insure that each of the arms has produced sufficient evidence of efficacy to warrant continuation of the effort to confirm the response and survival found in the pilot studies. The interim analysis does not compare regimens within the study except as relevant to safety.

The major purpose of this study is to compare the 4 regimens for impact on survival, overall toxicity, unique toxicity, and the relationship of toxicity to therapeutic benefit. The study endpoints for the individual treatments will be the rate of objective tumor regression, the proportion of complete and partial responses, duration of responses, overall survival, and the relationship of each endpoint to the level of side effects (therapeutic index). Toxicities will be considered in 3 separate groups: hematologic, gastrointestinal, and other. Minor objectives will include assessment of putative prognostic criteria: site of indicator lesion, distribution of disease (liver only, other only, or liver and other), degree of liver dysfunction, and individual laboratory tests. An assessment of the relationship between response and survival will utilize time-dependent covariate methods.

RESULTS

Through December 1985, a total of 149 patients had been randomized into all 4 arms. Of these 25 have died. The average patient has been followed for 4 months. In this preliminary analysis, 91 were evaluated for response. Of these 23 (25%) responded, 1 completely and 22 partially. Six of these patients subsequently progressed. An additional 30 (33%) of the patients have neither responded nor progressed. Thirty-eight patients (42%) progressed without response. Additional currently stable patients may respond.

These results are 2½ times better than prior GITSG experience (*23*). The combined response rate and the stable disease rate are consistent with the results obtained in the pilot studies. Survival which can only be estimated is within the expected range. The study has successfully passed the first stopping point analysis.

There has been no unanticipated severity or incidence of toxicities. There were 2 treatment deaths: 1 was due to leukopenia with FUra loading alone and 1 was due to renal failure with high-dose LV. Life-threatening hematologic toxicities occurred in 3 patients: 2 with high-dose LV weekly and 1 with low-dose LV weekly. Severe granulocytopenia occurred in 11 patients: 6 were with FUra alone, 4 were with escalation of LV (intermittent high-dose FUra), and 1 was with low-dose LV weekly. FUra alone with escalation produced the greatest incidence of severe or worse white blood cell count toxicity by 2-fold (30% vs. 14%).

There were also 16 cases of mucosal toxicities that were severe or worse. Five were oral and 11 were gastrointestinal. Ten occurred with high-dose weekly LV and 6 with low-dose weekly LV.

There were two severe neurotoxicities: 1 with low-dose LV weekly, and 1 with high-dose LV weekly. There were none with FUra alone or intermittent high-dose FUra.

DISCUSSION

Preliminary findings appear consistent with those of earlier phase II studies of biochemical modulation with LV in which rates of response and survival were superior to historical and contemporary experience in the collaboratives (*23–25*). The overall 2½-fold improvement in response rates is unusual for GITSG studies. In the past, GITSG consistently reported lower rates of response compared with single-institution or collaborative group findings.

This preliminary analysis found sufficient objective response and toxicity with the low-dose LV regimen to encourage further comparative testing. In the current studies, the larger weekly dosage of LV (500 mg/m^2) tended to produce more gastrointestinal toxicity, especially diarrhea, than the 25 mg/m^2 dosage.

The finding that low-dose LV with weekly low-dose FUra was possibly more toxic than high-dose FUra alone historically (dosage-intensive weekly treatments) and produced more severe gastrointestinal toxicity than FUra in this study (loading followed by escalation) provides confirmatory evidence of the biologic effect of LV. In contrast, a study of high-dose weekly intermittent FUra regimens with maximum doses of 20 mg/kg of FUra was closed early because the regimen, unlike the current weekly LV and FUra regimens, appeared to have a poor therapeutic index (*26*).

There appear to be qualitative differences, a reduced frequency of limiting hematologic toxicity, and increased distal mucosal toxicities with both low- and high-dose weekly LV regimens compared to the FUra regimen of loading with escalation. Finding more gastrointestinal than hematologic toxicity is, in theory, encouraging both in that the target of therapy are cells of gastrointestinal origin and in that it is easier to build on regimens which do not have limiting bone-marrow toxicity.

Escalating intermittent doses of LV and FUra are also being evaluated. Preliminary data suggests a substantially improved therapeutic index as well as savings in cost, compared with both intensive and standard treatments. Initially, this regimen appeared to be the least toxic regimen. The selective granulocytopenias have been brief and produced no serious clinical complications. The current regimens employ less frequent treatments with high-dose FUra. This appears to largely circumvent the historical problem of neurotoxicity associated with weekly or loading regimens using dosages larger than 1 g (total dose) of FUra (*26*).

It is still too early to compare these regimens. Initial similarities may be misleading. There may yet prove to be significant differences in response related to dose of LV which can be relevant to future adjuvant trials. Comparisons of survival and toxicities will be needed in order to define the regimen of choice for advanced disease trials. In theory, the best rate of response will be the critical factor in selecting an adjuvant regimen while best therapeutic index may be most important for the patient with advanced disease.

The dose and schedule of LV will require further study. Phase II regimens employing intermediate bolus doses of LV (200 mg/m^2 given immediately before bolus FUra in 5-day loading schedules) also appear to be producing similar high rates of response (*27,28*) as well as improved survival compared to historical experience. Similarly, LV (500 mg/m^2) as a 24-hour infusion for 6 days improves the response and the progression-free intervals compared with FUra given on days 2 to 6 as a bolus (*21,22*).

The current GITSG trial provides the only test comparing regimens employing LV for biochemical modulation of FUra to a maximally toxic FUra (escalation) control. Other controlled trials are only now increasing their starting dosage of FUra. The current GITSG trial is using FUra regimens of sufficient intensity to test the postulated relationship between the increased degree of side effect and increased therapeutic benefit of FUra alone. It can answer the question: Does LV improve the therapeutic index of FUra and produce additional benefit which cannot be achieved safely by escalating the dosage of FUra alone?

ADDENDUM

A 1987 toxicity review found that despite precautionary warnings, the weekly high-dose LV regimen was significantly more toxic for patients over 70 years of age than was any other regimen. The first course of treatment appears to carry nearly a 10% risk of fatal toxicities, beginning with mild gastrointestinal symptoms and, with further treatment, progressing to gastrointestinal bleeding, dehydration with or without renal failure, and septicemia. Therefore 1) the high-dose regimen is no longer recommended for the elderly, 2) high-dose treatment is now delayed at the first symptoms of gastroenteritis, 3) investigators recommend more intensive observation and earlier use of supportive care (fluids), and 4) adjuvant trials will begin at 500 rather than at 600 mg/m^2 of FUra.

REFERENCES

(*1*) Ansfield R, Klotz J, Nealon T, et al: A phase III study comparing the clinical utility of four regimens of 5-fluorouracil. Cancer 39:34–40, 1977.

(*2*) Mayer RJ, MacIntyre JM, Steele GD Jr, et al: Escalating bolus dose of 5-fluorouracil (5FU) for metastatic colorectal carcinoma. Proc Am Soc Clin Oncol 21:415, 1980.

(*3*) Mayer RJ, MacIntyre JM, Steele CD Jr, et al: High-dose bolus 5-fluorouracil (5FU) for metastatic colorectal carcinoma. Proc Am Soc Clin Oncol 2:127, 1983.

(*4*) Bruckner HW, Storch JA, Holland JF: Leucovorin increases the toxicity of 5-fluorouracil: Phase I clinical pharmacological trials. Proc Am Assoc Cancer Res 22:192, 1981.

(*5*) Bruckner HW, Ohnuma T, Hart R, et al: Leucovorin (LV) potentiation of 5-fluorouracil (FU) efficiency and potency. Proc Am Assoc Cancer Res 23:111, 1982.

(*6*) Bruckner HW, Roboz J, Spigelman M, et al: An efficient leucovorin and 5-fluorouracil sequence: Dosage escalation and pharmacological monitoring. Proc Am Assoc Cancer Res 23:138, 1983.

(*7*) Bruckner HW, Roboz J, Spigelman M, et al: An efficient leucovorin–5-fluorouracil sequence dosage escalation and pharmacologic monitoring. *In* Advances in Cancer Chemotherapy. The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: Park Row, 1984, pp 49–53.

(*8*) Bukowski RM, Cunningham J: Efficacy of 5-fluorouracil in combination with leucovorin in patients with colorectal carcinoma: Continuous infusion of low doses. *In* Advances in Cancer Chemotherapy. The Current Status of 5-Fluorouracil-Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: Park Row, 1984, pp 69–72.

(*9*) Cunningham J, Bukowski R, Budd T, et al: 5-Fluorouracil and folinic acid: A phase I-II trial in gastrointestinal malignancy. Invest New Drugs 2:391–395, 1984.

(*10*) Santi DV, McHenry CS, Sommer H: Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. Biochemistry 13:471–481, 1974.

(*11*) Ullman B, Lee M, Martin DW Jr, et al: Cytotoxicity of 5-fluoro-2′-deoxyuridine: Requirement for reduced folate cofactors and antagonism by methotrexate. Proc Natl Acad Sci USA 75:980–983, 1978.

(*12*) Waxman S, Bruckner HW, Wagle A, et al: Potentiation of 5-fluorouracil (5FU) antimetabolic effect by leucovorin (LV). Proc Am Assoc Cancer Res 19:149, 1978.

(*13*) Lockshin A, Danenberg PV: Biochemical factors affecting the tightness of 5-fluorodeoxyuridylate binding to human thymidylate synthetase. Biochem Pharmacol 30:247–257, 1981.

(*14*) Houghton JA, Maroda SJ Jr, Phillips JO, et al: Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinomas in mice. Cancer Res 41:144–149, 1981.

(*15*) Houghton JA, Houghton PJ: Basis for the interaction of 5-fluorouracil and leucovorin in colon adenocarcinoma. *In* Advances in Cancer Chemotherapy. The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: Park Row, 1984, pp 23–32.

(*16*) Evans RM, Laskin JD, Hakala MT: Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. Cancer Res 41:3288–3295, 1981.

(*17*) Berger SH, Hakala MT: Relationship of dUMP and free PdUMP pools to inhibition of thymidylate synthetase by 5-fluorouracil. Mol Pharmacol 25:303–309, 1984.

(*18*) Hakala MT: Basis for the effect of leucovorin on the activity and site of action of 5-fluorouracil in human cells. *In* Advances in Cancer Chemotherapy. The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: Park Row 1984, pp 33–39.

(*19*) Rustum YM, Madajewicz S, Campbell J, et al: Clinical biochemical pharmacology of 5-formyltetrahydrofolate in combination with 5-fluorouracil in patients with advanced colorectal carcinoma: Correlation with clinical response. *In* Advances in Cancer Chemotherapy. The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: Park Row, 1984, pp 77–82.

(*20*) Madajewicz S, Petrelli N, Rustum YM, et al: Phase I-II trial of high-dose calcium leucovorin and 5-fluorouracil in advanced colorectal cancer. Cancer Res 44:4667–4669, 1984.

(*21*) Bertrand M, Doroshow JH, Multhauf P, et al: Combination chemotherapy with high-dose continuous infusion leucovorin and bolus 5-fluorouracil in patients with advanced colorectal cancer. *In* Advances in Cancer Chemotherapy. The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: Park Row, 1984, pp 73–75.

(*22*) Bertrand M, Doroshow J, Multhauf P, et al: High dose folinic acid (HDFA) by continuous infusion and iv bolus 5-FU in patients with advanced colorectal cancer: A randomized study. Proc Am Soc Clin Oncol 4:77, 1985.

(*23*) Smith SP, Ellenberg SS, Mayer R, Lessner HE, Horton J, for the Gastrointestinal Tumor Study Group: Phase II study of MOF-streptozotocin (MeCCNU, vincristine, 5-fluorouracil and streptozotocin) in advanced colorectal cancer. Clin Oncol 2:770–773, 1984.

(*24*) Sugarbaker PH, Macdonald JS, Gunderson LL: Colorectal cancer. *In* Cancer. Principles and Practice of Oncology. (DeVita VT Jr, Hellman S, Rosenberg SA, eds). Philadelphia: JB Lippincott Co, 1982, pp 643–723.

(*25*) Schein P, Levin B: Neoplasms of the colon. *In* Medical Oncology. Basic Principles and Clinical Management of Cancer (Calabresi P, Schein PS, Rosenberg SA, eds). New York: Macmillan, 1985, pp 884–901.

(*26*) Horton J, Olson KB, Sullivan J, Reilly C, Schneider B. The Eastern Cooperative Oncology Group: 5-Fluorouracil in cancer: An improved regimen. Ann Intern Med 73:897–900, 1970.

(*27*) Machover D, Schwarzenberg L, Goldschmidt E, et al:

Treatment of advanced colorectal and gastric adenocarcinomas with 5FU combined with high-dose folinic acid: A pilot study. Cancer Treat Rep 66:1803–1807, 1982.

(*28*) MACHOVER D, TIMUS M, SCHWARZENBERG L, et al: Treatment of advanced colorectal and gastric adenocarcinomas with 5-fluorouracil combined with high-dose leucovorin: An update. *In* Advances in Cancer Chemotherapy. The Current Status of 5-Fluorouracil-Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: Park Row, 1984, pp 55–64.

Clinical Studies of Biochemical Modulation of 5-Fluorouracil by Leucovorin in Patients With Advanced Colorectal Cancer by the North Central Cancer Treatment Group[1] and Mayo Clinic[2]

Michael J. O'Connell,[3,*] David J. Klaassen,[4] Lloyd K. Everson,[5] Stephen Cullinan,[6] and Harry S. Wieand[3,7]

ABSTRACT—The North Central Cancer Treatment Group (NCCTG) and Mayo Clinic are collaborating in an ongoing, prospective, randomized clinical trial of new approaches to the chemotherapy of advanced metastatic colorectal cancer. Single agent 5-fluorouracil (FUra) given by intensive-course rapid intravenous administration serves as a control. Included among the experimental treatments are two regimens consisting of FUra plus leucovorin (folinic acid). One of these regimens uses folinic acid at a dose level of 200 mg/m^2 daily for 5 days based on earlier studies by Machover et al. (*4*). The second regimen uses folinic acid at 1/10 the dose level (20 mg/m^2 daily for 5 days), since this lower dose of folinic acid has been shown to produce peak serum levels equivalent to the concentration of folinic acid required in culture medium to produce optimal inhibition of L1210 cells by FUra in vitro, and because of the great expense of folinic acid when given at the higher dose levels. As of January 1986, 78 patients had been randomized to receive treatment with FUra alone or one of the FUra-folinic acid regimens. The toxicity of the folinic acid regimens has been clinically tolerable, with stomatitis and, to a lesser extent, diarrhea being dose-limiting. Hematologic toxicity has been very mild. There is suggestive evidence that folinic acid given at the higher dose level in combination with FUra at a constant dose produces more severe effects on the oropharyngeal mucosa. Preliminary tumor response and survival data remain blinded in accordance with NCCTG policy. Further patient accrual and follow-up are required to assess the therapeutic effect of these folinic acid regimens compared to FUra given alone.—NCI Monogr 5:185–188, 1987.

There is currently no systemic chemotherapy approach, using either single agents or combinations of cytotoxic drugs, which has been shown to be of substantial value in the treatment of advanced colorectal carcinoma. The fluorinated pyrimidines, with FUra being the most widely studied, have produced objective tumor responses, in our hands, under the best of circumstances among only approximately 20% of patients treated, with no improvement in survival. Thus, given the current paucity of treatment alternatives for these patients and limitations of empirically derived combination chemotherapy, there is considerable clinical interest in selectively enhancing the therapeutic effectiveness of FUra using the principles of biochemical modulation.

A variety of biochemical and cell culture studies in murine L1210 (*1*), sarcoma 180, human carcinoma HEP-2 (*2*), and xenografts of human colorectal carcinoma cell lines (*3*) have suggested that an excess of intracellular reduced folates is necessary for optimal inhibition of thymidylate synthetase and for an increased cytotoxic effect of the fluorinated pyrimidines.

Machover et al. (*4*) have reported encouraging results from a pilot study in a group of 30 advanced colorectal cancer patients treated with folinic acid (200 mg/m^2 iv bolus) immediately followed by FUra in a dose of 370 mg/m^2 (iv bolus) daily for 5 days with a 21-day drug-free interval between treatments. Objective tumor responses were reported in 9 of 16 previously untreated patients (56%) and 3 of 14 patients (21%) resistant to chemotherapy with FUra given either as a single agent or combined with other drugs. In a more recent update of their data, Machover et al. have reported objective tumor responses in 41% of 59 previously untreated patients with advanced colorectal cancer and 16% of 25 patients whose tumors were resistant to FUra (*5*).

Whereas Machover et al. elected to use folinic acid at a dose of 200 mg/m^2 in combination with FUra, the optimal dose of folinic acid required to enhance FUra in the treatment of human colorectal cancer is not known. Ullman et al. (*1*) have demonstrated optimal inhibition of murine leukemia L1210 cells by fluorinated pyrimidines in culture when the folinic acid content of the medium was approximately 1×10^{-6} *M*. A review of the pharmacologic litera-

---

ABBREVIATION: NCCTG = North Central Cancer Treatment Group.

---

[1] Participating institutions: Fargo Clinic (Fargo, North Dakota), Illinois Oncology Research Association (Peoria, Illinois), Mayo Clinic (Rochester, Minnesota), Duluth Clinic (Duluth, Minnesota), Central Plains Clinic (Sioux Falls, South Dakota), Saskatoon Cancer Clinic (Saskatchewan, Canada), University of Nebraska Medical Center (Omaha, Nebraska), Creighton University (Omaha, Nebraska), Iowa Oncology Research Association (Des Moines, Iowa), Grand Forks Clinic (Grand Forks, North Dakota), Quain and Ramstad Clinic (Bismarck, North Dakota), Billings Clinic (Billings, Montana), St. Cloud Internists, Ltd (St. Cloud, Minnesota), Rapid City Medical Center (Rapid City, South Dakota).

[2] Supported by grants CA-25224, CA-37404, CA-31224 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[3] Department of Oncology, Mayo Clinic, Rochester, MN.

[4] Saskatoon Cancer Clinic, University of Saskatchewan, Canada.

[5] Fargo Clinic, Fargo, ND.

[6] Illinois Oncology Research Association, Peoria, IL.

[7] We thank Lederle Laboratories (Pearl River, NY) for supplying folinic acid for this clinical trial.

* *Reprint requests to:* Michael J. O'Connell, M.D., Department of Oncology, Mayo Clinic, Rochester, Minnesota 55905.

ture (*6–10*) indicates that peak serum folinic acid levels greater than 1×10^{-6} *M* can be achieved in humans following the iv injection of less than 20 mg/m². Furthermore, the peak serum levels of folinic acid and its major metabolite, 5-methyltetrahydrofolic acid, do not increase in proportion to dose administered, but rather demonstrate some degree of plateauing at higher doses.

These scientific issues, in addition to the great expense of administering folinic acid at a dose of 200 mg/m² daily for 5 days (currently in excess of $2,000 per course in the United States), have led us to study folinic acid in combination with FUra at 2 distinct dose levels: 200 mg/m² based on the preliminary reports by Machover et al. (*4*), and a much lower dose (20 mg/m²) which should still yield peak serum levels of folinic acid equivalent to concentrations demonstrated to enhance FUra cytotoxicity in a murine model system.

We are currently studying these treatment concepts as part of a prospective, randomized clinical trial in which intensive-course FUra, given by rapid iv injection, serves as a control. After stratification according to performance status and presence of measurable malignant disease, patients are randomly assigned to receive treatments with 1 of 6 treatment regimens: FUra alone, methotrexate followed by FUra in 2 different administration schedules, FUra plus cisplatin, and FUra plus folinic acid at 2 different dose levels as described above. This report will describe eligibility, treatment methodology, statistical objectives, and preliminary toxicity observations for patients treated with FUra alone or FUra plus folinic acid at 1 of the dose levels under investigation. Tumor response and survival data from this ongoing trial remain blinded in accordance with NCCTG policy.

PATIENTS AND METHODS

Patient eligibility.—Patients must have histologic or cytologic confirmation of unresectable or metastatic colorectal cancer, with the site of origin of the primary lesions confirmed radiologically or surgically to be in the large bowel. The single exception to the requirement for histologic confirmation may be made in the patient who has had a previously histologically confirmed, primary colorectal carcinoma and a clinical diagnosis of pulmonary metastasis with no other tumor area accessible to biopsy. Under these circumstances, strict criteria must be met, including presence of 2 or more nodular lesions on chest x-ray that have either newly appeared or shown progression over a period of at least 1 month. All patients must be ambulatory and maintaining a reasonable state of nutrition (1,200 calories or greater daily oral intake). Patients with either measurable indicator lesions or nonmeasurable malignant disease are eligible. If liver metastasis is used as a measurable site of malignant disease, the liver must be palpable at least 5 cm below the costal margin, or a defect measuring at least 5 cm in diameter must be clearly defined using liver scan or CT scan. Patients with leukopenia (WBC count less than 4,000 cells per mm³), thrombocytopenia (platelet count less than 130,000 cells per mm³), serum creatinine levels above institutional normal limits, evidence of active infection, or frequent vomiting are not eligible for study. Nonambulatory patients and those with recent major surgery are also ineligible. Only patients who have not had previous exposure to chemotherapy are eligible for this trial. Patients with clinically significant, third space fluid accumulation and those with congestive heart failure are also excluded.

Table 1.—Chemotherapy administration schedules

Regimen	Drug	Dose and route	Days	Repeat cycle[a]
1	FUra	500 mg/m² iv	1–5	5 weeks
2	Folinic acid	200 mg/m² iv	1–5	4 weeks twice,
	FUra	370 mg/m² iv	1–5	then 5 weeks
3	Folinic acid	20 mg/m² iv	1–5	4 weeks twice,
	FUra	370 mg/m² iv	1–5	then 5 weeks

[a] Intervals between cycles measured from day 1 of each course of treatment.

Chemotherapy administration schedules.—FUra and folinic acid are each given by rapid iv injection on an outpatient basis. When folinic acid and FUra are given in combination, FUra is given immediately following administration of folinic acid. Table 1 summarizes the chemotherapy administration schedules. For all treatment regimens, the dose of FUra is escalated by 10% per treatment cycle in the absence of toxicity from the preceding cycle of therapy.

Statistical objectives.—The primary purpose of our overall randomized clinical trial is to evaluate the 5 experimental regimens compared to FUra alone to determine whether any of the experimental regimens yield improved survival. We will also assess comparative regression probabilities in the patients with measurable disease. We anticipate accruing 70 patients per treatment arm, with approximately 40 having measurable indicator lesions. The primary study end point is patient survival using a 1-sided log-rank test of statistical significance. It should be possible to detect an improvement in median survival from 6 months with FUra alone to 12 months with any of the experimental regimens with a power of 0.90 and a level of significance of 0.05. An improvement of the objective tumor response rate from 20% with FUra alone to 45% with any of the experimental regimens should be detected with a power of 0.80 and a level of significance of 0.05. We plan to provide for possible extension of selected treatment arms if further data seem desirable to establish possible treatment differences.

RESULTS

Administrative Data

Table 2 summarizes the status of patients randomized to receive FUra alone or one of the folinic acid plus FUra regimens as of January, 1986. Note that only 2 of the 78 patients randomized (2.6%) have been excluded from analysis because of ineligibility, cancellation, or improper treatment administration.

Toxicity

Hematologic toxicity is currently evaluable for 259 treatment cycles among 54 patients. The median number of cycles per patient is 3, with a range of 1 to 13 cycles per patient. As illustrated in table 3, single-agent FUra has produced a higher incidence of severe leukopenia compared to either of the folinic acid regimens. Only 4 of 42 patients (9.5%) receiving treatment with either of the folinic acid

TABLE 2.—Administrative data—January 1986

Patients	No. of patients in treatment regimen FUra alone	FUra + folinic acid, 200 mg/m^2	FUra + folinic acid, 20 mg/m^2	Total
Randomized	26	25	27	78
Ineligible, cancelled, or disqualified	0	0	2	2
Properly entered and treated	26	25	25	76
Measurable indicator lesion	13	13	13	39
Currently evaluable for toxicity	23	21	21	65

regimens have had WBC counts less than 2,000 cells per mm^3 at any time during treatment. Significant thrombocytopenia has not been seen following the first cycle of therapy with any of the regimens, and platelet counts below 75,000 cells per mm^3 have been seen in only 3 of 63 patients (5%) at any time during treatment. There have been no treatment-related fatalities.

Nonhematologic toxicity has consisted primarily of stomatitis and diarrhea as reviewed in table 4. Note that 6 of 21 patients (29%) receiving FUra plus folinic acid at the 200 mg/m^2 dose level experienced clinically severe stomatitis compared to 2 of 21 patients (10%) treated with FUra plus folinic acid at 20 mg/m^2. It should also be noted that 9 of 21 patients (43%) receiving folinic acid at the 20 mg/m^2 dose level had an increase in FUra dose on subsequent treatment cycles because of lack of toxicity, but only 2 of 13 patients (15%) treated with folinic acid at 200 mg/m^2 who received more than one treatment cycle could have their FUra dose increased.

DISCUSSION

The preliminary reports by Machover et al. (*4*) of the activity of folinic acid plus FUra in the treatment of advanced colorectal cancer are of definite interest, but clearly require confirmation by controlled clinical trial. We are currently evaluating this concept using 2 distinct dosage levels of folinic acid since the optimal dose of folinic acid to produce enhancement of FUra in the treatment of human colorectal cancer is not known. In the absence of mature data from prospective randomized trials comparing folinic acid regimens to single-agent FUra, it is not possible at this time to state what impact, if any, this new treatment approach will have on tumor response or patient survival.

TABLE 3.—Hematologic toxicity

	Treatment regimen FUra alone	FUra + folinic acid, 200 mg/m^2	FUra + folinic acid, 20 mg/m^2
	% of 22 patients	% of 20 patients	% of 22 patients
WBC nadir, cells/mm^3			
<2000	36 (50)[a]	5 (15)	5 (5)
2000–4000	45 (41)	45 (35)	50 (75)
Platelet nadir, cells/mm^3			
<75,000	0 (9)	0 (5)	0 (0)
75,000–130,000	18 (18)	0 (0)	5 (14)

[a] The first number indicates percent of patients with respective blood count nadir following the first cycle of chemotherapy. The number in parentheses indicates percent of patients whose lowest blood count nadir was in indicated range at any time on study.

TABLE 4.—Severe nonhematologic toxicity[a] (worst overall)

	Treatment regimen FUra alone, % of 23 patients	FUra + folinic acid, 200 mg/m^2 % of 21 patients	FUra + folinic acid, 20 mg/m^2 % of 21 patients
Stomatitis	17	29	10
Diarrhea	9	14	14

[a] Severe stomatitis (painful oropharyngeal ulcerations which significantly interfered with food intake) or diarrhea (more than 4 watery bowel movements daily).

Our preliminary observations indicate that the regimens of folinic acid plus FUra we are studying are clinically tolerable and that mucocutaneous side effects rather than hematologic toxicity are dose-limiting. Our data suggest that folinic acid given at 200 mg/m^2 (daily for 5 days) in combination with FUra at a constant dose level (370 mg/m^2 daily for 5 days) produces more severe effects on the oropharyngeal mucosa compared to folinic acid given at 20 mg/m^2 (daily for 5 days). Whether this apparent enhancement of FUra toxicity will also translate into a greater antitumor effect remains to be seen. Because of our goal to evaluate each of the treatment regimens in our randomized clinical trial using the cytotoxic agents near the maximal doses that are clinically tolerable in order to afford the greatest opportunity for tumor response, we have increased the dose of FUra to 425 mg/m^2 daily for 5 days in combination with folinic acid at the lower dose level (20 mg/m^2 daily for 5 days). We have maintained the FUra dose at 370 mg/m^2 in combination with folinic acid at the 200 mg/m^2 dose level for 5 days.

It is our hope that data from this and other ongoing controlled clinical trials will eventually put the concept of folinic acid–FUra chemotherapy for advanced colorectal cancer into perspective and afford some insight concerning optimal dose levels and administration schedules of these two agents in combination.

REFERENCES

(*1*) ULLMAN B, LEE M, MARTIN DW, et al: Cytotoxicity of 5-fluoro-2′-deoxyuridine: Requirement for reduced folate cofactors and antagonism of methotrexate. Proc Natl Acad Sci USA 75:980–983, 1978.

(*2*) EVANS RM, LASKIN JD, HAKALA MT: Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. Cancer Res 41:3283–3295, 1981.

(*3*) HOUGHTON JA, MAROD SJ JR, PHILIPS JO, et al: Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinomas in mice. Cancer Res 41:144–149, 1981.

(*4*) MACHOVER D, SCHWARZENBERG L, GOLDSCHMIDT E, et al: Treatment of advanced colorectal and gastric adenocarcinomas with 5-FU combined with high-dose folinic acid: A pilot study. Cancer Treat Rep 66:1803–1807, 1982.

(*5*) Machover D, Goldschmidt E, Chollet P, et al: Treatment of advanced colorectal and gastric adenocarcinoma with 5-FU and high dose folinic acid. Proc Am Assoc Cancer Res 26:175, 1985.

(*6*) Mehta BM, Gisolfi AL, Hutchison DJ, et al: Serum distribution of citrovorum factor and 5-methyltetrahydrofolate following oral and IM administration of calcium leucovorin in normal adults. Cancer Treat Rep 62:345–350, 1978.

(*7*) Mehta BM, Glass JP, Shapiro WR: Serum and cerebrospinal fluid distribution of 5-methyltetrahydrofolate after intravenous calcium leucovorin and intra-ommaya methotrexate administration in patients with meningeal carcinomatosis. Cancer Res 43:435–438, 1983.

(*8*) Rothenberg SP, Da Costa M, Rosenberg Z: A radiochemical assay for N^5-formyltetrahydrofolic acid (citrovorum factor) in serum and urine. Anal Biochem 93:483–487, 1979.

(*9*) Spray GH, Witts LJ: The utilisation of folinic acid injected intravenously. Clin Sci 12:391–397, 1953.

(*10*) Hamel E, Johnson G, Glaubiger D: Pharmacokinetics of leucovorin rescue using a new methotrexate-independent biochemical assay for leucovorin and N^5-methyltetrahydrofolate. Cancer Treat Rep 65:545–553, 1981.

Biologic Modulation of 5-Fluorouracil With High-Dose Leucovorin and Combination Chemotherapy of 5-Fluorouracil and Cisplatin in Metastatic Colorectal Adenocarcinoma[1]

Nicholas J. Petrelli,[2,*] Stefan Madajewicz,[3] Lemuel Herrera,[2] Youcef M. Rustum,[4] Fabio Trave,[4] Patrick Creaven,[3] and Arnold Mittelman[2]

ABSTRACT—The data from an ongoing 3-arm prospective study of 72 patients with advanced colorectal carcinoma is presented. The 3 regimens are as follows: Regime 1 (every 4 weeks)—5-fluorouracil (FUra) (450 mg/m^2 iv bolus daily for 5 days, then 200 mg/m^2 iv bolus every other day for 6 doses); regime 2 (every week for 4 weeks, then every other week)—methotrexate (MTX) (50 mg/m^2 in a 4-hour infusion) followed by FUra (600 mg/m^2 iv bolus); regime 3 (weekly for 6 weeks followed by a 2-week rest period)—D,L-leucovorin (D,L-CF) (500 mg/m^2 in a 2-hour infusion) with FUra (600 mg/m^2 iv bolus) 1 hour after the D,L-CF infusion began. All monitoring lesions except lung were documented by tissue biopsy. Thirteen of 18 patients in the FUra + D,L-CF arm were evaluable for response. Six of the 13 patients (46%) have had a partial response. The duration of the 6 responses has been 11, 8, 7, 4, 3 and 3 months. In patients with liver metastases as the monitoring lesion, a dramatic improvement in liver function tests has been seen during the first 2 courses (12 weeks) of treatment, but this was not sustained. The toxicity of FUra + D,L-CF was predominantly gastrointestinal; unlike with FUra alone, myelosuppression was not predominant.—NCI Monogr 5:189–192, 1987.

FUra, used alone, produces an objective response in 15% to 20% of patients with advanced colorectal cancer (*1*). Various early combination studies have yielded results that are not superior to those with FUra alone. Recently, attempts have been made to enhance the effectiveness of FUra based on considerations of the biochemical pathways of FUra activation and its site of action. Two mechanisms of the antitumor effect of FUra have been proposed: 1) activation to 5-fluorouridine triphosphate and incorporation into RNA; and 2) activation to 5-fluorodeoxyuridine monophosphate (5-FdUMP) with competitive inhibition of thymidylate synthase (TS) by formation of a ternary complex with the active site of TS and 5,10-methylenetetrahydrofolic acid (5,10-CH_2FH_4). Modulation of the first mechanism can be achieved by coadministration of thymidine which reduces the catabolism of FUra, thus increasing its bioavailability. However, in clinical trials, thymidine increased the toxicity of FUra but did not affect its therapeutic efficacy (*2–4*). D,L-CF has been utilized to modulate FUra via the second mechanism (*5–13*). Evans et al. (*6*) have shown in cell culture that the stability of the FdUMP-TS-5,10-CH_2FH_4 ternary complex is maximal when the extracellular concentration of D,L-CF is 20 μM. Similar results were reported by Houghton et al. (*14*).

This report reviews the phase I (toxicity evaluation), II (therapeutic efficacy), and III (response comparison) studies of FUra and high-dose D,L-CF at Roswell Park Memorial Institute in patients with advanced colorectal adenocarcinoma. It also discusses the future metabolic modulation studies to be performed, specifically the modulation of FUra by high-dose D,L-CF and cisplatin in view of a completed toxicity and therapeutic efficacy study of combination FUra and cisplatin. The latter data are presented.

MATERIALS AND METHODS

All patients in the FUra and D,L-CF trial and the FUra and cisplatin trial had histologic documentation of primary colorectal adenocarcinoma. All distant metastatic sites except pulmonary were also histologically documented. Prior to each course of therapy, every patient had a complete blood count, a serum electrolyte determination, liver-function tests, a serum creatinine, a blood urea nitrogen, a chest x-ray, a liver scan, and an abdominal and pelvic computer-assisted tomography scan or ultrasound where appropriate. During each course, the complete blood count, liver function tests, creatinine, and blood urea nitrogen were repeated weekly.

Patients undergoing FUra and D,L-CF therapy were considered evaluable for toxicity if they received at least 1 course (6 weekly doses of FUra and D,L-CF). Patients were evaluated for response after 2 courses (12 doses). Standard response criteria were used to evaluate antitumor effect: a) complete response—complete disappearance of all recognizable tumor masses; b) partial response—a 50% reduction in the product of the largest perpendicular diameters of the most clearly measurable area of known malignant disease, no increase in the size of other measurable disease, and no appearance of new lesions; c) stable—a decrease in

---

ABBREVIATIONS: 5,10-CH_2FH_4 = 5,10-methylenetetrahydrofolic acid; D,L-CF = leucovorin; 5-FdUMP = 5-fluorodeoxyuridine monophosphate; MTX = methotrexate; TS = thymidylate synthase.

---

[1] Supported by Lederle Laboratories.

[2] Department of Surgical Oncology, Roswell Park Memorial Institute, Buffalo, NY.

[3] Department of Clinical Pharmacology and Therapeutics, Roswell Park Memorial Institute, Buffalo, NY.

[4] Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, NY.

[*] *Reprint requests to:* Nicholas J. Petrelli, M.D., Department of Surgical Oncology, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263.

tumor size less than partial response with no appearance of new lesions; and d) progression—an increase in a measurable lesion by greater than 25% and/or the appearance of any new lesions.

RESULTS

Toxicity and Therapeutic Efficacy of FUra and High-Dose D,L-CF

Twenty-six patients with metastatic colorectal adenocarcinoma were entered into a phase I–II study of FUra and high-dose D,L-CF at Roswell Park Memorial Institute. The starting dose of FUra was 300 mg/m^2 (with escalation to 750 mg/m^2/week in 6 doses) given by rapid iv injection midway during a 2-hour infusion of D,L-CF (500 mg/m^2). Partial responses were seen in 9 of 23 patients (6 of 12 who had previous FUra treatment). Complete normalization of liver enzymes was seen in 2 of these patients. Side effects were seen sporadically with FUra doses up to 600 mg/m^2. At 600 mg/m^2 of FUra, 8 of 18 patients had diarrhea and 2 of 18 had WBC counts $<$3000 mm^3. At a 750-mg/m^2 dose of FUra, 6 of 11 patients had severe diarrhea and 6 of 11 had WBC counts $<$3000 mm^3. Other toxicities were mild conjunctivitis and lacrimation, thinning of the nails, and alopecia. The recommended phase III dose was 600 mg/m^2 of FUra and 500 mg/m^2 of D,L-CF. These patients have been included in the denominator for a subsequent randomized phase III trial of FUra and D,L-CF versus FUra alone versus FUra and MTX, with a response rate of 40% in 18 patients on the FUra and D,L-CF arm (table 1).

A total of 72 patients (54 males and 18 females) have been randomized into the ongoing phase III prospective study: regime 1 (every 4 weeks)—FUra (450 mg/m^2 iv bolus for 5 days, then 200 mg/m^2 iv bolus every other day for 6 doses); regime 2 (every week for 4 weeks, then every two weeks)—MTX (50 mg/m^2) in a 4-hour infusion followed by FUra (600 mg/m^2 iv bolus); regime 3 (every week for 6 weeks, followed by a 2-week rest period)—D,L-CF (500 mg/m^2 in a 2-hour infusion) with FUra (600 mg/m^2 iv bolus) 1 hour after the D,L-CF has begun. The monitoring lesions were: FUra and MTX: liver 12, lung 7, pelvic 1, abdominal mass 2; FUra: liver 11, lung 6, axillary, and pelvic and inguinal masses, 1 each; FUra and D,L-CF: liver 11, lung 6, pelvic mass 1. Responses are seen in table 1. The duration of the 6 responses in the FUra and D,L-CF regime have been 11, 8, 7, 4, 3, and 3 months. Toxicity requiring a drug-dose reduction is illustrated in table 2. The toxicity of FUra and D,L-CF is predominately diarrhea and, unlike with FUra alone, myelosuppression is not predominant. All toxicity of FUra and D,L-CF has been reversible with a dose-reduction of FUra to 500 mg/m^2.

Toxicity and Therapeutic Efficacy of Combination FUra and Cisplatin

Combination chemotherapy is used with the hope that 1 or more of the assets needed to produce therapeutic synergism will be present in the combination. These assets are: a) the drugs used in the combinations will have less than additive toxicity for vital normal cells; b) the drugs will have different biochemical mechanisms of cytotoxic activity for drug-sensitive and -resistant tumor cells; c) tumor cells resistant to 1 or more drugs used in treatment will be sensitive to one or more other drugs in the combination; and/or d) the drugs used in combination will modulate each other's metabolism, resulting in a new potent killing effect. Data of Schabel et al. (*15*) indicate rather marked therapeutic synergism between cisplatin and FUra, since 60% of the animals treated with the drug combination were cured and the median lifespan of the combination-treated mice dying of leukemia exceeded the median lifespan of those treated with either cisplatin or FUra alone. A phase II study by Einhorn et al. (*16*) employing cisplatin (60 mg/m^2) every 4 weeks for 6 weeks and FUra (15 mg/kg by iv push) weekly showed a response rate of 11 partial responses and 1 complete response out of 38 patients with metastatic colorectal carcinoma. This study is important because, as a single agent, cisplatin is an ineffective drug for metastatic colorectal carcinoma.

TABLE 1.—Responses: Phase III

Regimen (No./patients)	Partial response	Stable	Progression
FUra (18)	2 (11%)	0	16 (88%)
FUra + MTX (21)	1 (5%)	2 (9%)	18 (85%)
FUra + D,L-CF (13)	6 (46%)	3 (23%)	4 (30%)

TABLE 2.—Toxicity: Phase III

Regimen (No. of patients)	Stomatitis	Diarrhea	Leukopenia	Thrombocytopenia
FUra (19)	2 (10%)	5 (26%)	8 (42%)	0
FUra + MTX (22)	2 (9%)	0	9 (41%)	1
FUra + D,L-CF (18)	0	4 (22%)	3 (16%)	0

In a study at Roswell Park Memorial Institute using FUra and cisplatin to determine toxicity and therapeutic efficacy, 24 patients have been evaluated to date (*17*). Cisplatin and FUra were given as a continuous infusion for 5 days as follows: cisplatin was begun at 20 mg/m^2 in 1000 ml of normal saline over 20 hours with FUra at 600 mg/m^2 in 1000 ml of 5% glucose in 0.5 *N* saline over 20 hours, followed by a 4-hour flush with 500 ml of 5% glucose in 0.5 *N* saline plus 20 milliequivalents of KCl. This regime was given each day for 5 days and was considered 1 course. Twenty-four patients had documented metastatic colorectal adenocarcinoma, and all patients had been treated with prior chemotherapy. Responses were as follows: 1 complete response, 2 partial responses, 4 stable, and 17 progressions. All patients were evaluated after 2 courses of chemotherapy. Myelosuppression was seen in 1 patient with a WBC count of 1,900/mm^3. Renal toxicity was seen in 4 patients with an increased serum creatinine. If the creatinine clearance was $<$50 ml per minute at the time of the next course of treatment but the serum creatinine was $<$1.5 g/100 ml, the dose of cisplatin was decreased by 5 mg/m^2. If the serum creatinine was $>$1.5 g/100 ml, chemotherapy was withheld until the serum creatinine became $<$1.5 g/100 ml.

Future Study

The purpose of this presently developing protocol at Roswell Park Memorial Institute is to enhance the responses seen with the combination of FUra (600 mg/m^2) and high-dose D,L-CF (500 mg/m^2) by the addition of cisplatin (fig. 1). Because of the low doses of cisplatin that will be used, severe nausea and vomiting are not anticipated;

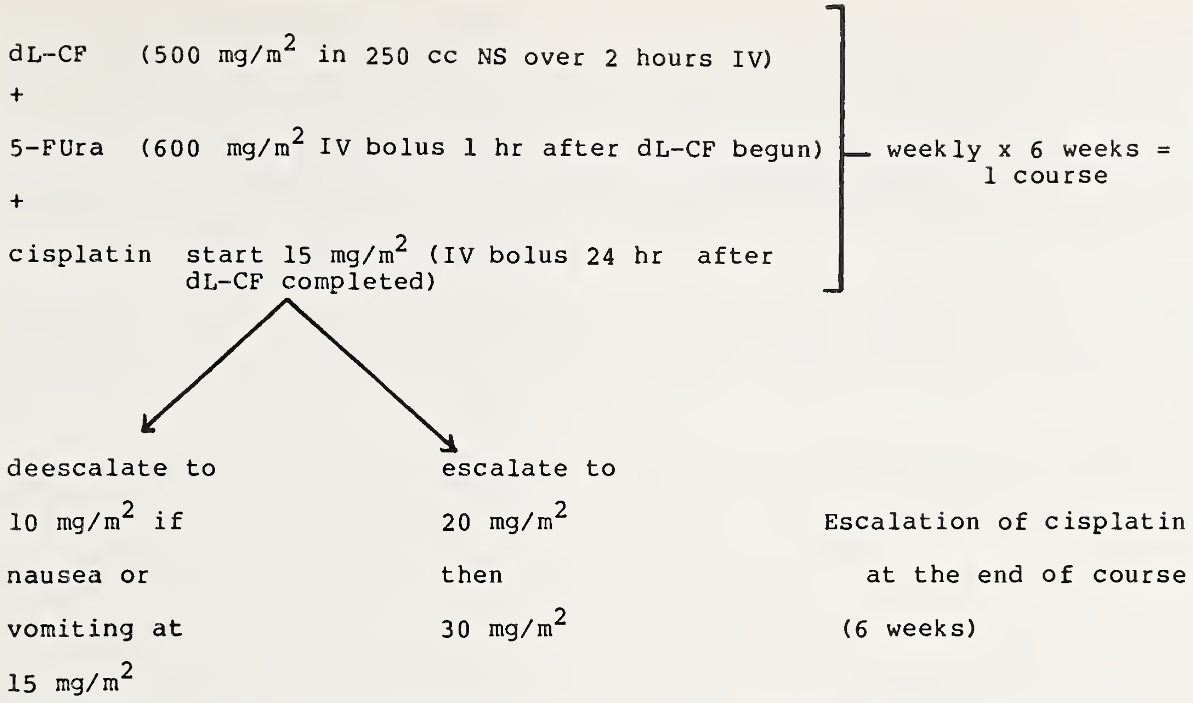

dL-CF (500 mg/m2 in 250 cc NS over 2 hours IV)
+
5-FUra (600 mg/m2 IV bolus 1 hr after dL-CF begun)
+
cisplatin start 15 mg/m2 (IV bolus 24 hr after dL-CF completed)
weekly x 6 weeks = 1 course
deescalate to 10 mg/m2 if nausea or vomiting at 15 mg/m2
escalate to 20 mg/m2 then 30 mg/m2
Escalation of cisplatin at the end of course (6 weeks)

FIGURE 1.—Schema for the toxicity and therapeutic efficacy protocol of FUra, cisplatin, and high-dose leucovorin.

therefore, an iv bolus dose of cisplatin will be used instead of a continuous infusion.

DISCUSSION

Thymidine rescue and protection studies have revealed that the inhibition of TS by 5-FdUMP is the primary site of the growth-inhibitory action of FUra in many, although not all, cells (*5,11*). According to the work of Ullman et al. (*18*), the levels of folates which were sufficient for the normal growth of L1210 cells did not provide maximal binding of 5-FdUMP to TS. These authors demonstrated a 3-fold increase in sensitivity of L1210 cells to 5-fluorouridine when the D,L-CF content of the medium rose from 0.01 to 1.0 μM or that of folic acid from 2.8 to 23 μM. It appears that the rate of recovery of TS activity plays a more significant role in the potency and site of action of FUra than does the amount of 5-FdUMP that is formed, and that this recovery can be greatly retarded with an excess of folate or D,L-CF (*10,19*).

A phase I study of this combination was carried out by Bruckner et al. (*20*). Low-dose D,L-CF (25 mg every 8 hr) was combined with FUra (1100 mg/m^2/day) by continuous infusion for up to 4 days with moderate toxicity. In a later study by Bruckner et al. (*21*), there was an indication of a better response to this combination than to FUra alone. The combination of FUra with high-dose D,L-CF has been published by Machover et al. (*12*). FUra (370 to 400 mg/m^2 daily for 5 days) was combined with D,L-CF (200 mg/m^2 daily for 5 consecutive days). Only 7 episodes of marked granulocytopenia (WBC count $<$1000 mm^3) were observed in 210 courses, and a response rate of $>$50% was seen in previously untreated patients with colorectal carcinoma, which was considerably higher than that seen with FUra alone. An update of this data is presented by Drs. Bruckner and Machover in this symposium.

These data strongly suggest that simultaneous administration of FUra and high-dose D,L-CF may enhance the antitumor activity of FUra in colorectal tumors. Other ongoing prospectively randomized studies are discussed in this symposium.

REFERENCES

(*1*) LEVIN P, MITTELMAN A, DOUGLASS H, et al: Survival and response to chemotherapy for advanced colorectal adenocarcinoma: An Eastern Cooperative Oncology Group Report. Cancer 46:1536–1543, 1980.

(*2*) YOON JM, BRUNO S, LEDESMA E, et al: A Phase I–II study of thymidine plus 5-FU continuous infusion chemotherapy in advanced colorectal carcinoma. Proc Am Assoc Cancer Res, ASCO 21:157, 1980.

(*3*) STERNBERG A, PETRELLI NJ, AU JL, et al: A combination of 5-fluorouracil and thymidine in advanced colorectal carcinoma. Cancer Chemother Pharmacol 13:218–222, 1984.

(*4*) AU JLS, RUSTUM YM, LEDESMA EJ, et al: Clinical pharmacological studies of concurrent infusion of 5-fluorouracil and thymidine in treatment of colorectal carcinomas. Cancer Res 42:2930–2937, 1982.

(*5*) EVANS RM, LASKIN JD, HAKALA MT: Assessment of growth limiting events caused by 5-fluorouracil in mouse cells and in human cells. Cancer Res 40:4113–4122, 1980.

(*6*) EVANS RM, LASKIN JD, HAKALA MT: Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. Cancer Res 41:3283–3295, 1981.

(*7*) MADAJEWICZ S, PETRELLI NJ, RUSTUM Y, et al: A phase I trial of high dose leucovorin (CF) and 5-fluorouracil (5-FU) in advanced colorectal cancer. Cancer Res 44:4667–4669, 1984.

(*8*) MACHOVER D, SCHWARZENBERG L, GOLDSCHMIDT E, et al: Treatment of advanced colorectal and gastric adenocarcinomas with 5-fluorouracil (5-FU) combined with high dose folinic acid (FA). Proc Am Assoc Cancer Res 23:33, 1982.

(*9*) SANTI DV, MCHENRY CS, SOMMER H: Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. Biochemistry 13:471–480, 1974.

(*10*) Rustum YM, Madajewicz S, Campbell J, et al: Clinical biochemical pharmacology of 5-formyltetrahydrofolate in combination with 5-fluorouracil in patients with advanced colorectal carcinoma: Correlation with clinical response. *In* Advances in Cancer Chemotherapy—The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: J. Wiley & Sons, 1984, pp 77–82.

(*11*) Hakala MT: Basis for the effect of leucovorin on the activity and site of action of 5-fluorouracil in human cells. *In* Advances in Cancer Chemotherapy—The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: J. Wiley & Sons, 1984, pp 33–39.

(*12*) Machover D, Goldschmidt E, Schwarzenberg L, et al: Treatment of advanced colorectal and gastric adenocarcinoma with FUra combined with high-dose leucovorin: An update. *In* Advances in Cancer Chemotherapy—The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: J. Wiley & Sons, 1984, pp 55–64.

(*13*) Yin MB, Zakrzewski S, Hakala MT: Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. Mol Pharmacol 23:190–197, 1983.

(*14*) Houghton JA, Marod SJ Jr, Phillips JO, et al: Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenograft of human colorectal adenocarcinoma in mice. Cancer Res 41:144–149, 1982.

(*15*) Schabel FM Jr, Tader MW, Laster WR, et al: *cis*-Dichlorodiammineplatinum(II): Combination chemotherapy and cross-resistance studies with tumors of mice. Cancer Treat Rep 63:1459–1473, 1979.

(*16*) Einhorn LH, Williams D, Loehrer P: Combination chemotherapy with platinum (P) plus 5-FU in metastatic colorectal carcinoma. Proc ASCO 3:133, 1984.

(*17*) Madajewicz S, Trave F, Petrelli N, et al: Preclinical and clinical studies with 5-fluorouracil (FU) and cisplatin (DDP). Proc ASCO 4:47, 1985.

(*18*) Ullman B, Lee M, Martin DW Jr, et al: Cytotoxicity of 5-fluoro-2′-deoxyuridine: Requirement for reduced folate cofactors and antagonism by methotrexate. Proc Natl Acad Sci USA 75:980–983, 1978.

(*19*) Berger SE, Hakala MT: Relationship of dUMP and free FdUMP pools to inhibition of thymidylate synthetase by 5-fluorouracil. Mol Pharmacol 25:303–309, 1984.

(*20*) Bruckner HW, Ohnuma T, Hart R, et al: Leucovorin (LV) potentiation of 5-fluorouracil (FU) efficiency and potency. Proc Am Assoc Cancer Res 23:111, 1982.

(*21*) Bruckner HW, Storch JA, Holland JF: Leucovorin increases the toxicity of 5-fluorouracil: Phase I clinical pharmacological trials. Proc Am Assoc Cancer Res 22:111, 1982.

Treatment of Advanced Colorectal and Gastric Adenocarcinomas With 5-Fluorouracil and High-Dose Folinic Acid[1]

David Machover,[2,*] Emma Goldschmidt,[2] Philippe Chollet,[3] Gérard Metzger,[2] Jacqueline Zittoun,[4] Manuel Benavides,[2] Jeanine Marquet,[4] Jean-Marie Vandenbulcke,[2] Jean-Louis Misset,[2] Léon Schwarzenberg,[2] Jean-Pierre Lotz,[2] Jean B. Fourtillan,[5] Hélène Gaget,[2] and Georges Mathé[2,6]

ABSTRACT—We report the results of an expanded trial of 5-fluorouracil (FUra) combined with high-dose folinic acid for treatment of patients with advanced colorectal or gastric adenocarcinoma. In each treatment course, the patients received both FUra (340-400 mg/m^2/day by iv infusion over 15 minutes) and folinic acid (200 mg/m^2/day by iv bolus) for 5 consecutive days, with a 21-day interval between courses. Eighty-six patients with colorectal carcinoma were evaluated. The combined complete response (CR) and partial response (PR) rates were 39% for 54 patients who did not receive prior chemotherapy and 22% for 32 patients who had previously received chemotherapy. Four patients who were previously resistant to FUra attained objective responses. The median time to disease progression for the 28 responders was 10 months. The median survival time of responders was 19.5 months, and the probability of their being alive at 2 years was 40%. Of 27 patients with gastric adenocarcinoma, 13 (48%) responded to therapy. Their median time to disease progression was 5.5 months. The median survival time of responders was 11 months, and their probability of being alive at 15 months was 30%. Toxicity was within acceptable limits. Toxic effects included stomatitis, diarrhea, conjunctivitis, skin rash, and mild myeloid hypoplasia. In a separate study, plasma concentrations of L-folates above 10^{-5} *M* were achieved after a rapid single iv injection of 200 mg/m^2 of folinic acid. Comparisons of our results with those reported in previous studies on FUra given as a single agent suggest that, in advanced colorectal and gastric adenocarcinoma, folinic acid administered in high doses may enhance the effectiveness of FUra given concomitantly. Furthermore, some colorectal tumors which were previously resistant to FUra become sensitive to the drug. The survival of the patients who responded to therapy was markedly improved over that observed in reported series of untreated patients with advanced colorectal and gastric adenocarcinomas.—NCI Monogr 5:193-198, 1987.

In an effort to improve the antineoplastic activity of 5-fluorouracil (FUra) in colorectal and gastric adenocarcinomas with visceral metastases and/or massive regional extension, we designed a clinical trial in which we combined this fluorinated pyrimidine and high-dose folinic acid. The rationale for this clinical trial will be presented during this symposium.

Preliminary data on the clinical trial described here, which was begun in December 1979, have already been reported (*1*). We are now presenting an extension of the study, in which both the number of patients entered and the follow-up time were increased. We also present plasma pharmacokinetic data obtained after iv administration of folinic acid at the dose used in this clinical trial.

MATERIALS AND METHODS

All of the patients entered in this study had histologically confirmed colorectal or gastric adenocarcinoma, and all had metastatic disease. Patients previously untreated with cytostatic agents as well as patients who had received prior chemotherapy of any type were eligible to enter the trial. Patients considered evaluable had objectively measurable disease, including clearly defined, palpable tumor masses or parenchymal nodules which could be measured by means of imaging procedures. Serosal effusions, bone metastases, and abnormal findings in biologic tests (including tests for carcinoembryonic antigen levels) were not considered as indicating measurable disease.

Patients With Colorectal Adenocarcinoma

Ninety-two patients were entered in the study; 6 of these were excluded because their tumors were not accurately measurable. Of the 86 evaluable patients, 73 had measurable widespread or multiple hepatic metastases. Of these, 27 had other sites of malignancy as well, and 46 had metastases confined to the liver only. Of 32 patients who had previously received chemotherapy, 27 had been treated ineffectively for advanced disease with FUra given either as a single agent or combined with other drugs; all the patients in this group had unequivocal evidence of progression while treated with FUra and were thus considered resistant to the drug. Prior resistance to FUra could not be demonstrated in the other 5 previously treated patients for various reasons: 2 patients had received chemotherapy without FUra for advanced disease, and 3 patients had relapsed after completion of adjuvant treatment with FUra. The remaining 54 patients had not received prior chemotherapy.

ABBREVIATIONS: CR = complete response; PR = partial response; CHOFH$_4$ = formyltetrahydrofolic acid; CH$_3$FH$_4$ = methyltetrahydrofolate; HPLC = high-pressure liquid chromatography; NC = no change; PD = progressive disease; $t_{1/2}$ = half-life.

[1] Supported by the Association Claude-Bernard and by the Association pour la Recherche sur le Cancer (ARC).

[2] Service des Maladies Sanguines et Tumorales & ICIG (Univ. Paris-Sud, CNRS LA-149, Assoc. Cl.-Bernard & ARC), Hôpital Paul-Brousse, 94804 Villejuif, France.

[3] Centre Jean-Perrin, 63 Clermont-Ferrand, France.

[4] Service Central d'Hématologie-Immunologie, Hôpital Henri Mondor, 94 Créteil, France.

[5] CEROM, 86 Poitiers, France.

[6] We acknowledge the secretarial assistance of Mrs. Nicole Vriz and Christiane Grin.

* *Reprint requests to:* David Machover, M.D., Service d'Oncologie Médicale, Hôpital Tenon, 4, Rue de la Chine, 75970 Paris Cedex 20, France.

Patients With Gastric Adenocarcinoma

Thirty patients were entered in the study; 3 of these were excluded because their tumors could not be measured accurately. Of the 27 patients with measurable disease who were evaluated (table 1), 1 had previously received ineffective treatment that included administration of FUra; this patient was considered resistant to the drug. The other 26 patients had not received previous chemotherapy.

Treatment

Therapy consisted of 5-day courses of folinic acid and FUra followed by drug-free intervals of 21 days. Folinic acid was given in a dose of 200 mg/m^2/day by iv bolus injection. Immediately afterwards, FUra was administered at a dose of 370 mg/m^2 by iv infusion over 15 minutes. In subsequent courses, the daily dose of FUra was raised to 400 mg/m^2 if toxicity was either absent or mild at 370 mg/m^2. The daily dose of FUra was reduced by 30 mg/m^2 in cases of granulocytopenia (less than 1000 polymorphonuclear leukocytes/mm^3) complicated by infection, and/or in the presence of grade 3 or 4 (World Health Organization) oral stomatitis or diarrhea (*2*). Courses were repeated until treatment failure became evident in nonresponding patients. In previously responding and previously stable patients, cycles of treatment were repeated until massive local or extensive systemic tumor growth developed, except for 1 responder whose treatment was discontinued after the eighteenth course at his request. Responses were categorized according to World Health Organization criteria (*2*).

Statistical Methods

Survival times were calculated from the start of therapy. Time to disease progression was calculated from the date of initiation of therapy to the date when progressive disease was first observed. Plots were constructed according to the method of Kaplan and Meier (*3*), comparisons were performed with the log-rank test, and 95% confidence intervals were calculated by the method of Rothman (*4*).

Plasma Pharmacokinetics of Folates

Folinic acid is a chemically synthesized form of reduced folate which consists of equal amounts of the diastereoisomers D- and L-5-formyltetrahydrofolic acid (CHOFH$_4$). It has been established that the L form can be transformed in vivo into active folate cofactors (*5,6*). However, it is possible that the unnatural D-isomer affects the cellular biology of folates (*6–9*). In the present study, we determined the plasma kinetics of both the D and L forms of folinic acid and that of folate metabolites resulting from the transformation of L-5-CHOFH$_4$ after a single iv injection of the drug.

Subjects.—Six patients with normal hepatic function, normal creatinine clearance, and no clinically diagnosed visceral failure were selected for study. They had no prior history of folate therapy or of cytostatic chemotherapy.

Protocol.—Folinic acid (Lederfoline, Lederle Laboratories, 94-Rungis, France), at a dose of 200 mg/m^2 of body surface, was dissolved in 20 ml of 5% glucose solution and injected iv over 5 minutes. Blood samples were drawn prior to drug administration and at 5, 10, 15, 20, 30, and 45 minutes and 1, 2, 4, 8, 12, and 24 hours after the iv injection. Blood samples were collected in heparinized tubes to which ascorbic acid (1 mg/ml of blood) was added; then the tubes were centrifuged in the cold. The plasma was stored at −20° C until analyzed.

Determination of plasma levels of D,L-5-CHOFH$_4$.—Acetonitrile (2 ml) and 6% trichloroacetic acid (0.1 ml) were added to 1 ml of plasma; the mixture was shaken and centrifuged. The supernatant was added to dichloromethane (7 ml) and centrifuged; then the aqueous layer (0.8 ml) was passed through a QAE Sephadex column. Folinic acid was eluted from the resin with 1 *M* acetic acid (0.5 ml), and the

TABLE 1.—Advanced gastric adenocarcinoma: response to therapy according to patient characteristics

Characteristic	Response to therapy: No. of patients (%)				
	CR	PR	NC[a]	PD[b]	Total No.
All patients	1 (3.7)	12 (44.4)	4 (14.8)	10 (37.0)	27
Prior chemotherapy					
No	1 (3.8)	12 (46.2)	4 (15.4)	9 (34.6)	26
Yes	—	—	—	1	1[c]
Sites of tumor					
Liver only	—	1	3	1	5
Liver and other sites[d]	—	5	—	2	7
Abdominal tumor only	—	1	1	2	4
Abdominal tumor and other sites[e]	1	1	—	2	4
Lung and mediastinum only	—	3	—	—	3
Stomach only	—	1	—	2	3
Performance status					
0–1	—	1	—	—	1
2	—	5	3	3	11
3	—	6	—	6	12
4	1	—	1	—	2

[a] NC = no change.

[b] PD = progressive disease.

[c] This patient was resistant to previously administered FUra.

[d] Including stomach, lymph nodes, abdomen, and abdominal wall.

[e] Including stomach and lung.

eluate was adjusted to pH 6 with 1 *N* sodium hydroxide. Samples of 100 μl of this solution were injected into a high-performance liquid chromatography (HPLC) system. The apparatus consisted of a Waters Associates pump, model 6000A, and a WISP 710B injector; the detector was a UV 160 model (Beckman) set at 280 nm. Total D,L-5-CHOFH$_4$ was measured by ion-pair chromatography. The column used was an ODS 5 μm ultrasphere (Altex), and the mobile phase was methanol (26%) and acetic acid (0.5%) in water, adjusted to pH 5.5. Measurements were made by the external-standard method. The detection limit of the assay was 0.5×10^{-6} *M.*

Assay for L*-folates.*—The quantity of folates in their L-forms was measured on 3 bacterial strains. *Pediococcus cerevisiae* (ATCC 8081) was used for the measurement of L-5-CHOFH$_4$, *Lactobacillus casei* (ATCC 7469) for all L-folate compounds, and *Streptococcus faecalis* (ATCC 8043) for all L-folates except methyltetrahydrofolate (L-5-CH$_3$FH$_4$) (*5,6*). To determine growth of the microorganisms, we measured the amount of lactic acid produced after 48 hours of culture at 37° C; the lactic acid was titrated against 0.1 *N* sodium hydroxide with an automatic pH meter. The detection limit of the assay was 5×10^{-10} *M.* Concentrations of D-5-CHOFH$_4$ in plasma were calculated as the difference between the concentration of D,L-5-CHOFH$_4$ measured by HPLC and that of L-5-CHOFH$_4$ determined by the *P. cerevisiae* assay. Plasma concentrations of all L-folates resulting from the metabolism of L-5-CHOFH$_4$ were calculated as the difference between the concentrations of all L-folate forms as measured by the *L. casei* assay and those of L-5-CHOFH$_4$ as determined by the *P. cerevisiae* assay. Concentrations of L-5-CH$_3$FH$_4$ were calculated as the difference between the concentrations of all L-folate forms as measured by the *L. casei* assay and the concentrations of all L-folates except L-5-CH$_3$FH$_4$ as measured by the *S. faecalis* assay.

Data analysis.—The data on plasma concentration (C) versus time (t) for the various folates, as obtained directly and by difference, were fitted to the equation $C(t) = Ae^{-\alpha t}$ and $C(t) = Be^{-\beta t}$ by the method of least squares to yield the elimination rate constants (α and β) and the half-lives ($t\frac{1}{2}$) for each subject.

RESULTS

Patients With Colorectal Carcinoma

All Patients

Of the 86 evaluable patients, 4 attained CR and 24 PR (table 2). The overall response rate was thus 33% (95% confidence interval, 0.23 to 0.43).

During the observation period, 3 patients died of causes not related to tumor development. Three patients were lost to follow-up with no evidence of disease progression. These 6 patients were included as censored at the time of death or at the time of the last follow-up.

The times to disease progression for the responders ranged from 2 to 40.9+ months (median, 10 months). The median survival time for the 28 responders was 19.5 months. Their probability of being alive at 2 years was 40%, with a 95% confidence interval of 0.22 to 0.6. Twenty-five of all the colorectal carcinoma patients had no change in their disease; their median survival time was 13 months, and their probability of being alive at 2 years was 18%.

No Previous Chemotherapy

Of the 54 patients who had not previously received chemotherapy, 2 achieved CR and 19 PR (table 2), yielding a

TABLE 2.—Advanced colorectal adenocarcinoma: Response to therapy according to patient characteristics

Characteristic	Response to therapy: No. of patients (%)				
	CR	PR	NC	PD	Total No.
Patients without prior chemotherapy					
All patients	2 (3.7)	19 (35.2)	11 (20.4)	22 (40.7)	54
Sites of tumor					
Liver	—	9	6	11	26
Liver and other sites[a]	—	8	2	11	21
Other sites[b]	2	2	3	—	7
Performance status					
0–1	—	8	7	9	24
2	2	7	4	10	23
3	—	4	—	3	7
Patients with prior chemotherapy					
All patients	2[c] (6.3)	5[c] (15.6)	14 (43.8)	11 (34.4)	32
Sites of tumor					
Liver	2	3	10	5	20
Liver and other sites[a]	—	—	2	4	6
Other sites[b]	—	2	2	2	6
Performance status					
0–1	1	3	5	3	12
2	1	1	7	5	14
3	—	1	2	1	4
4	—	—	—	2	2

[a] Including lung, abdomen, pelvis, adrenals, and peripheral nodes.

[b] Including pelvis, lung, peripheral nodes, and abdomen.

[c] Of these 7 responders, 4 had previous resistance to FUra; prior resistance to FUra could not be demonstrated in 3 patients. One of these 3 had received prior therapy for advanced disease without FUra, and 2 had relapsed after completion of adjuvant chemotherapy with FUra.

response rate in this group of 39% (95% confidence interval, 0.26 to 0.52). Responses lasted from 2 to 40.9+ months (median, 10 months). The median survival time of responders was 19.5 months, and their probability of remaining alive at 2 years was 32%, with a 95% confidence interval of 0.15 to 0.56. Eleven of the 54 patients had stable disease. Their median survival time was 17 months, and their probability of being alive at 2 years was 31% (fig. 1).

Previous Chemotherapy

Of the 32 patients who had previously received chemotherapy, 2 achieved CR and 5 PR (table 2); the response rate was 22% (95% confidence interval, 0.08 to 0.36). Of the 7 patients who responded to therapy, 4 (2 with CR and 2 with PR) were known to be resistant to previously administered FUra; the durations of their responses were 7, 10, 12, and 15 months, and their survival times were 11.4, 25, 18.5, and 26.9 months, respectively. The other 3 patients who responded to therapy had no demonstrable prior resistance to FUra; the durations of their responses were 5, 12.5, and 24+ months, and their survival times were 8.7, 13+, and 24+ months, respectively. Fourteen patients (44%) had no change in their disease; their median survival was 12 months, and their probability of being alive at 2 years was 17%.

Patients With Gastric Carcinoma

Of the 26 patients who had not previously received chemotherapy, 1 had CR and 12 PR (table 1), yielding a response rate in this group of 50%. The single previously treated patient did not respond to therapy. The overall response rate was thus 48% (95% confidence interval, 0.29 to 0.67).

The median survival time of the 13 responders was 11 months, and their probability of remaining alive at 15 months was 30%, with a 95% confidence interval of 0.11 to 0.6 (fig. 2).

Toxicity

As reported previously (*1*), the degree of acute myeloid toxicity was moderate, with rapid recovery, and no cumulative myelosuppression resulted from the treatment employed.

The 111 patients who were evaluable for toxic effects received a total of 874 courses of therapy (range per patient, 1 to 28 courses), 830 of which were evaluated for toxic effects. By dose level of FUra, 11 patients received 340 mg/m^2/day for 5 days; 111 patients, 370 mg/m^2/day for 5 days; and 77 patients, 400 mg/m^2/day for 5 days. Toxicity is summarized in table 3.

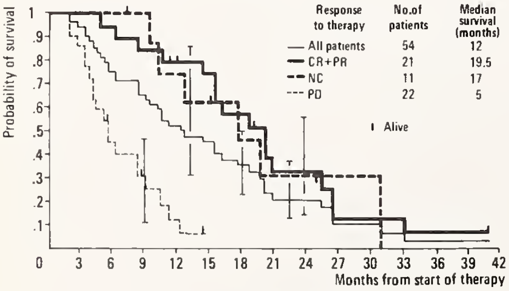


FIGURE 1.—Survival of patients with advanced colorectal carcinoma who had not received prior chemotherapy ($N=54$). Two patients died of causes not related to tumor development at 3.2 and 8.5 months from start of treatment, and 1 patient was lost to follow-up with no evidence of disease progression at 8.2 months from the start of therapy; they contributed to the curves as censored at their time of death or last follow-up. *Vertical bars* indicate the 95% confidence intervals.

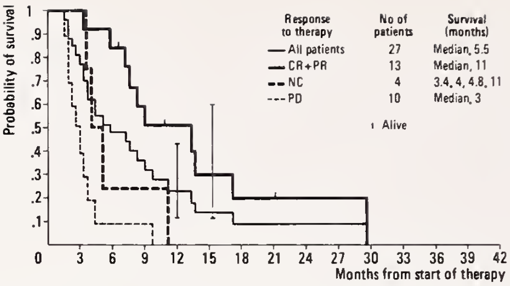


FIGURE 2.—Survival of all patients with advanced gastric carcinoma ($N=27$) according to response to therapy. Twenty-six patients had not received prior chemotherapy. The single patient with previous chemotherapy had progressive disease. *Vertical bars* indicate the 95% confidence intervals.

TABLE 3.—Toxic effects in patients treated with FUra and high-dose folinic acid

	No. of patients with toxic effects (%)[a]			
		According to dose of 5-FU (mg/m^2/day for 5 days):		
	Total	340	370	400
Toxic effect and degree	(111 patients)	(11 patients)[b]	(111 patients)[b]	(77 patients)[b]
Oral stomatitis[c]	49 (44)			
1		4 (36)	16 (14)	20 (26)
2		2 (18)	14 (13)	20 (26)
3		—	3 (3)	11 (14)
4		—	—	1 (1)
Diarrhea[d]	43 (39)			
1		1 (9)	8 (7)	10 (13)
2		2 (18)	17 (15)	13 (17)
3		—	5 (4)	3 (4)
4		—	—	2(3)
Fever[e]	13 (12)			
Alopecia[f]	12 (11)			
Dry skin rash	10 (9)			
Conjunctivitis and lacrimation	12 (11)			
Thoracic pain	1 (1)			

[a] All patients were evaluated. Two additional patients died of tumor progression within 2 weeks of the start of therapy and therefore could not be evaluated.

[b] Total number of patients by dose level of FUra.

[c] World Health Organization grades: 1 = soreness; 2 = ulcers, can eat solids; 3 = ulcers, requires liquid diet; and 4 = alimentation not possible.

[d] World Health Organization grades: 1 = transient, <2 days; 2 = tolerable, >2 days; 3 = intolerable, requires therapy; and 4 = dehydration.

[e] In 12 patients, the cause of fever was not documented microbiologically; in 1, it was due to *Staphylococcus aureus* septicemia. One patient died during an episode of diarrhea and fever not documented microbiologically. During febrile episodes, granulocytopenia (<10^3 polymorphonuclear cells/mm^3) was documented in 2 patients at the dose level of 370 mg/m^2/day for 5 days and in 6 at 400 mg/m^2/day for 5 days.

[f] Partial in 11 patients, complete in 1 patient.

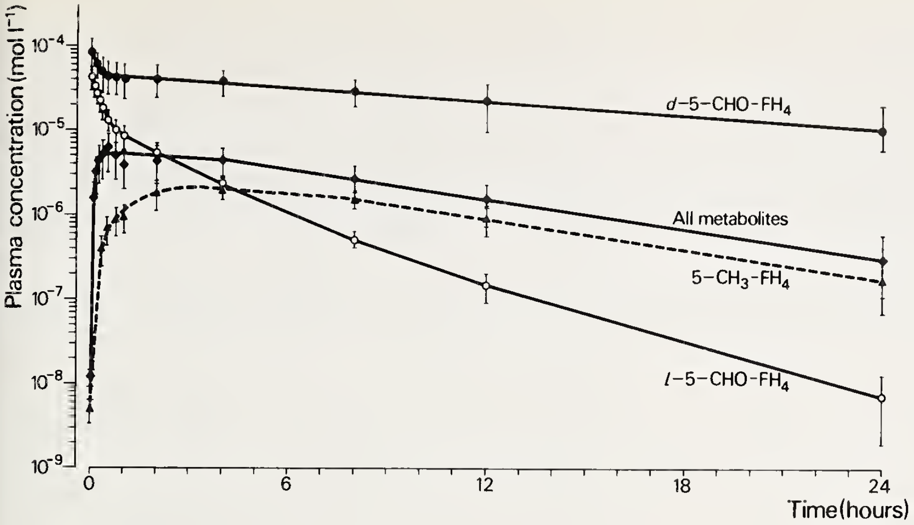


FIGURE 3.—Plasma concentration of folates (mean ± SE) vs. time, after a single iv injection of folinic acid at a dose of 200 mg/m^2 in 6 subjects. Values of t½β were 122 ± 20 min for L-5-CHOFH$_4$, 659 ± 100 min for D-5-CHOFH$_4$, 362 ± 59 min for L-CH$_3$FH$_4$, and 349 ± 57 min for total L-metabolites.

Toxic effects required FUra dose reductions in 9 patients (8%) at 370 mg/m^2/day, and in 21 patients (27%) at 400 mg/m^2/day. Severe toxicity, when present, was effectively prevented in subsequent courses by a 30 mg/m^2 decrease in the daily dose of FUra.

Plasma Pharmacokinetics of Folates

As shown in figure 3, the L-form of folinic acid (L-5-CHOFH$_4$) reached a mean maximal concentration of 4.3×10^{-5} *M;* it was rapidly cleared from the plasma with t½ α and t½ β values of 20 ± 2 minutes and 122 ± 20 minutes, respectively. Plasma concentrations of the natural isomer were maintained above 10^{-5} *M* for 1 hour from the time of injection. Large amounts of folate metabolites, consisting mainly of L-5-CH$_3$FH$_4$, appeared rapidly (within 5 to 10 minutes from the time of injection); they reached a mean maximal concentration of 5×10^{-6} *M* and were cleared from the plasma slowly: half-lives of 362 ± 59 minutes and 349 ± 57 minutes were measured for L-5-CH$_3$FH$_4$ and for all L-folate metabolites, respectively. The D isomer of 5-CHOFH$_4$ persisted in the plasma at concentrations greatly exceeding those of the L form. The plasma t½ β for the unnatural isomer was 659 ± 100 minutes.

DISCUSSION

Extensive studies on FUra for treatment of advanced colorectal carcinoma have been performed in the past. Data from various sources which differ by the numbers of patients treated and the doses and schedules of FUra employed (*10-12*) have indicated that the response rates obtained with this agent fluctuate widely. However, the mean response rate was 20%. The therapeutic efficacy of FUra has been questioned in 3 recent studies (*13-15*) in which the response rates obtained ranged only from 8% to 13%. Because the time to disease progression in the small number of responders was short and because survival was not increased appreciably, FUra treatment alone has been thought to have, at most, minimal therapeutic value in advanced colorectal carcinoma.

In the present trial, we observed a response rate of 39% in colorectal carcinoma patients who had not received prior chemotherapy. This result is superior to the responses reported in most series of patients who were treated primarily with FUra administered as a single agent (*10-15*). In patients who had previously received chemotherapy, the response rate was 22%. Moreover, objective responses were obtained in some patients whose tumors had previously been resistant to FUra. Other studies that consisted of combined FUra and high-dose reduced folates for treatment of patients with advanced gastrointestinal malignancies are also presented during this symposium.

Our data suggest that the treatment we employed increased the survival time of responders with metastatic colorectal carcinoma compared with that reported for the series of untreated patients (*16-20*). In these reports, the median survival times ranged from 2 to 10 months with an average of about 6 months, and the probability of survival at 2 years from the diagnosis of metastatic disease was below 10% (*16-20*). In the present trial, the median survival time of responders who had not previously received chemotherapy was 19.5 months, and their probability of being alive at 2 years from the start of therapy was 40%. Although the data obtained for patients with no change are more difficult to analyze, our results suggest that the induction of stable disease by the treatment used in the present study may also have a favorable impact upon survival.

For patients with advanced gastric carcinoma, treatment in the past with FUra given as a single agent yielded overall response rates of 20% to 25% (*21*). Durations of response were 4 to 5 months on the average, and only minor improvement in short-term survival was obtained (*22*). In the present study, 50% of the patients with gastric adenocarcinoma who had not received prior chemotherapy attained an objective response. The median survival of responding patients was 11 months, and their probability of being alive at 15 months from the start of treatment was 30%. These results appear to be better than those observed in most series of untreated patients with metastatic or unresectable gastric carcinoma (*22-24*), in which the reported median survival times have ranged from 2 to 4 months.

Comparisons of our results in both colorectal and gastric adenocarcinomas with those reported in the historical series mentioned above suggest that the addition of high doses of folinic acid may improve the therapeutic activity of FUra in these types of tumors. It remains clear, however, that the superiority of the combination of FUra and folinic acid over FUra alone will only be definitely demonstrated with the results of appropriate randomized trials.

The toxicity observed during our study was within acceptable limits, except that there was one therapy-related death. Oral stomatitis and diarrhea were the most prominent toxic effects. Although most episodes were mild or moderate, some severe stomatitis and diarrhea occurred, mainly at the higher doses of FUra.

The pharmacokinetic data presented here confirm that L-5-$CHOFH_4$ is rapidly cleared from the plasma, and that this clearance is accompanied by the formation of large amounts of folate metabolites (*6*). We have shown that a single rapid iv injection of folinic acid at 200 mg/m^2 results in plasma concentrations of L-folates above 10^{-5} *M*, the level required for maximal potentiation of fluoropyrimidines in the cell culture systems tested thus far (*25-27*). However, the relationship between plasma concentrations of folates and the attainment of the required intratumor levels of active folates is, as yet, unknown.

Our data also substantiate previous findings that the unnatural D-5-$CHOFH_4$ has a much longer half-life than does the L-isomer (*6*). The slow disappearance of the D-isomer from plasma should be considered in future studies, because there is competition between the diastereoisomers for carrier-mediated membrane transport (*7,8*). Moreover, the D-5-$CHOFH_4$ may interfere with the L-isomer for intracellular polyglutamation, because both diastereoisomers are substrates for mammalian folylpolyglutamate synthetase (*9,28*).

From the present study, we conclude that FUra combined with high-dose folinic acid provides an effective form of palliative treatment for patients with advanced colorectal or gastric adenocarcinoma. Therefore, we are extending this treatment to other tumors in which FUra has shown some therapeutic effectiveness, to the exploration of other treatment schedules for fluoropyrimidines and reduced folates, and to prospective adjuvant treatment of postsurgical minimal residual disease in patients with gastrointestinal adenocarcinoma.

REFERENCES

(*1*) Machover D, Schwarzenberg L, Goldschmidt E, et al: Treatment of advanced colorectal and gastric adenocarcinomas with 5-FU combined with high-dose folinic acid: A pilot study. Cancer Treat Rep 66:1803–1807, 1982.

(*2*) World Health Organization: Handbook for Reporting Results of Cancer Treatment. Geneva: World Health Organization, 1979.

(*3*) Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481, 1958.

(*4*) Anderson JR, Bernstein L, Pike MC: Approximate confidence intervals for probabilities of survival and quantiles in life-table analysis. Biometrics 38:407–416, 1982.

(*5*) Blakley RL: The Biochemistry of Folic Acid and Related Pteridines. *In* North-Holland Research Monographs, Frontiers of Biology, vol 13. Amsterdam: North-Holland, 1969.

(*6*) Straw JA, Szapary D, Wynn WT: Pharmacokinetics of the diastereoisomers of leucovorin after intravenous and oral administration to normal subjects. Cancer Res 44:3114–3119, 1984.

(*7*) White JC, Bailey BD, Goldman ID: Lack of stereospecificity at carbon 6 of methyltetrahydrofolate transport in Ehrlich ascites tumor cells. Carrier-mediated transport of both stereoisomers. J Biol Chem 253:242–245, 1978.

(*8*) Sirotnak FM, Chello PL, Moccio DM, et al: Stereospecificity at carbon 6 of formyltetrahydrofolate as a competitive inhibitor of transport and cytotoxicity of methotrexate in vitro. Biochem Pharmacol 28:2993–2997, 1979.

(*9*) Sato JK, Moran RG: Interaction of methotrexate (MTX) and citrovorum factor (CF) at folylpolyglutamate synthetase (FPGS). Proc Am Assoc Cancer Res 3:312, 1984.

(*10*) Moertel CG: Current concepts in cancer. Chemotherapy of gastrointestinal cancer. N Engl J Med 229:1049–1052, 1978.

(*11*) Livingston RB, Carter S: Single Agents in Cancer Chemotherapy. New York: IFI/Plenum, 1970, pp 195–226.

(*12*) Davis HL: Chemotherapy of large bowel cancer. Cancer 50:2638–2646, 1982.

(*13*) Presant CA, Denes AE, Liu C, et al: Prospective randomized reappraisal of 5-fluorouracil in metastatic colorectal carcinoma. A comparative trial with 6-thioguanine. Cancer 53:2610–2614, 1984.

(*14*) Windschitl H, Scott M, Schutt A, et al: Randomized phase II studies in advanced colorectal carcinoma: A North Central Cancer Treatment Group Study. Cancer Treat Rep 67:1001–1008, 1983.

(*15*) Engstrom PF, MacIntyre JM, Mittelman A, et al: Chemotherapy of advanced colorectal carcinoma: fluorouracil alone vs two drug combinations using fluorouracil, hydroxyurea, semustine, decarbazine, razoxane and mitomycin. A phase III trial by the Eastern Cooperative Oncology Group. Am J Clin Oncol 7:313–318, 1984.

(*16*) Bengmark S, Hafstrom L: The natural history of primary and secondary malignant tumors of the liver. I. The prognosis for patients with hepatic metastases from colonic and rectal carcinoma by laparotomy. Cancer 23:198–202, 1969.

(*17*) Moertel CG: Clinical management of advanced gastrointestinal cancer. Cancer 36:675–682, 1975.

(*18*) Wood CB, Gillis CR, Blumgart LH: A retrospective study of the natural history of patients with liver metastases from colorectal cancer. Clin Oncol 2:285–288, 1976.

(*19*) Cady B: Natural history of primary and secondary tumors of the liver. Semin Oncol 10:127–134, 1983.

(*20*) Lahr CJ, Soong SJ, Cloud G, et al: A multifactorial analysis of prognostic factors in patients with liver metastases from colorectal carcinoma. J Clin Oncol 1:720–726, 1983.

(*21*) Comis RL, Carter SK: Integration of chemotherapy into combined modality treatment of solid tumors. III. Gastric cancer. Cancer Treat Rev 1:221–238, 1974.

(*22*) Kovach JS, Moertel GC, Schutt AJ, et al: A controlled study of combined 1,3-bis-(2-chloroethyl)-l-nitrosourea and 5-fluorouracil therapy for advanced gastric and pancreatic cancer. Cancer 33:563–567, 1974.

(*23*) Buchholtz TW, Welch CE, Malt RA: Clinical correlates of resectability and survival in gastric carcinoma. Ann Surgery 188:711–715, 1978.

(*24*) Bedikian AY, Chen TT, Khankhanian N, et al: The natural history of gastric cancer and prognostic factors influencing survival. J Clin Oncol 2:305–310, 1984.

(*25*) Evans RM, Laskin JD, Hakala MT: Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. Cancer Res 41:3288–3295, 1981.

(*26*) Ullman B, Lee M, Martin DW Jr, et al: Cytotoxicity of 5-fluoro-2′-deoxyuridine: Requirement for reduced folate cofactors and antagonism by methotrexate. Proc Natl Acad Sci USA 75:980–983, 1978.

(*27*) Waxman S, Bruckner H: The enhancement of 5-fluorouracil antimetabolic activity by leucovorin, menadione and α-tocopherol. Eur J Cancer Clin Oncol 18:685–692, 1982.

(*28*) Moran RG: Characterization of the function of mammalian folylpolyglutamate synthetase (FPSG). Adv Exp Med Biol 163:327–339, 1983.

Preliminary Results of a Phase II Trial for the Treatment of Metastatic Breast Cancer With 5-Fluorouracil and Leucovorin

Carmen J. Allegra,* Bruce A. Chabner, Pam W. Sholar, Caroline Bagley, James C. Drake, and Marc E. Lippman[1]

ABSTRACT—The active metabolite of FUra, 5-fluorodeoxyuridine monophosphate (5-FdUMP), requires the presence of reduced folates to form a covalent ternary complex with the target enzyme thymidylate synthase (TS). In vitro and in vivo studies have demonstrated a potentiation of the cytotoxic effects of FUra when combined with the reduced folate, leucovorin. We have applied this concept to the treatment of metastatic breast cancer in a phase II trial, as recent clinical studies on patients with colorectal carcinoma have suggested an enhanced efficacy for the combination of FUra plus leucovorin. Patients entered on the present study are undergoing treatment with a 5-day daily regimen of leucovorin (500 mg/m^2, iv) followed by FUra (375 mg/m^2, iv). Toxicity and response data are currently being collected on patients who have failed "standard" combination regimens that included FUra. In patients with accessible tumor, serial biopsies are being obtained during treatment with the combination of FUra and leucovorin and during therapy with FUra alone to assess the degree of 5-FdUMP binding to the target enzyme, TS, in the presence and absence of exogenously administered leucovorin. Preliminary results from the biochemical studies suggest an enhanced saturation of TS by the fluorinated pyrimidine when administered with leucovorin.—NCI Monogr 5:199–202, 1987.

TS is a critical folate-requiring enzyme in the de novo synthesis of thymidylate, 1 of the 4 nucleotides required for DNA synthesis. The required substrates for the TS reaction include deoxyuridylate and the reduced folate, 5,10-methylenetetrahydrofolate. In addition to thymidylate, the reaction catalyzed by TS is the only known biological reaction that requires not only the transfer of a carbon group from the folate but also the transfer of two hydrogens yielding the oxidized folate, dihydrofolic acid. The production of this folate necessitates the presence of dihydrofolate reductase to reduce the dihydrofolate to the tetrahydrofolate required for continued function of TS and the folate-requiring enzymes in the de novo purine synthetic pathway. Inhibition of TS results in a "thymineless" state and ultimately cell death. The fluorinated pyrimidine, FUra, is capable of binding to and potently inhibiting the catalytic reaction (inhibition constant [K_i]=10^{-11} *M*) after conversion of the inactive parent compound to the active metabolite, 5-FdUMP (*1*). Several investigators have demonstrated that the binding of 5-FdUMP to TS (and therefore inhibition) requires the presence of the folate cosubstrate (*2,3*). Houghton et al. have demonstrated that colon carcinoma xenografts are capable of growing in folate concentrations that are suboptimal for the binding of FdUMP to TS and, as a result, are relatively resistant to the cytotoxic effects of FUra (*4*). Such cells can be made sensitive to the fluorinated pyrimidine by the addition of reduced folates to the growth media. Yin et al. have further demonstrated that FUra resistance in the HEP-2 human carcinoma line is associated with a low intracellular folate concentration (*5*). Extracellular concentrations of 10 μM L-5-formyltetrahydrofolic acid (leucovorin) have been reported to promote maximal binding of 5-FdUMP to TS in intact cells (*6*). In cell-free systems, using highly purified human TS, a concentration of 5×10^{-5} *M* of monoglutamated 5,10-methylenetetrahydrofolate is required for complete saturation of the enzyme by 5-FdUMP. However, 50- to 100-fold less of the naturally occurring polyglutamated folate, 5,10-methylenetetrahydrofolate pentaglutamate, (1×10^{-6} *M*) is needed for complete binding of the enzyme by 5-FdUMP (*7*) (table 1).

Preliminary clinical evidence suggests that the FUra-leucovorin combination may have enhanced activity in human carcinomas. A variety of doses and schedules utilizing the combination of FUra and leucovorin have been investigated for the treatment of advanced colorectal carcinoma (*8,9*). Machover et al., using a treatment schedule of 375 mg/m^2/day for 5 days preceded by a bolus of 200 mg/m^2/day of leucovorin, reported a 45% response rate in untreated patients and an 18% response rate in prior FUra failures (*8*). Using a regimen of 650 mg/m^2 weekly of FUra given at the midpoint of a 2 hour 500-mg/m^2 infusion of leucovorin, Petrelli reported an overall response rate of 38% for patients with colon carcinoma (*9*). For patients with advanced colorectal carcinoma, evidence for the enhanced effectiveness of the combination as compared to FUra alone is being investigated in ongoing randomized trials. However, measurements of target enzyme binding by the active metabolite in the tumor have been hampered by the apparent low enzyme activity in colorectal carcinoma and the relative difficulty in obtaining tissue samples in this disease.

We have initiated a phase II clinical trial for the treatment of metastatic breast carcinoma with the combination of FUra and leucovorin. The objectives of the study are to measure the response rate in a heavily pretreated population (including FUra failures) and to determine the effect of the combination on target enzyme binding through mea-

---

ABBREVIATIONS: 5-FdUMP=5-fluorodeoxyuridine monophosphate; K_i=inhibition constant; TS=thymidylate synthase.

[1] Clinical Pharmacology Branch and Medicine Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

* *Reprint requests to:* Carmen J. Allegra, M.D., National Cancer Institute, Bldg 10, Rm 6N119, Bethesda, MD 20892.

TABLE 1.—Formation of ternary complex with TS, 5-FdUMP, and 5,10-methylenetetrahydrofolate monoglutamate, and pentaglutamate

	% Maximal complex formation[a]
5,10-Methylenetetrahydrofolate monoglutamate (M)	
5×10^{-5}	100
8×10^{-6}	50
2×10^{-6}	20
5,10-Methylenetetrahydrofolate pentaglutamate	
1×10^{-6}	100
8×10^{-8}	50
2×10^{-8}	20

[a] Percent of maximal ternary complex formation is tabulated as a function of concentration of either 5,10-methylenetetrahydrofolate monoglutamate or pentaglutamate. Ternary complex was measured by allowing excess [6-^{3}H]5-FdUMP (3 pmol), 0.01 units of purified human TS (sp act 1.5 units/mg protein), 50 mM KH_2PO_4, pH 7.2, and various concentrations of monoglutamated or pentaglutamated 5,10-methylenetetrahydrofolate to reach an equilibrium, then quantitating the protein-bound [6-^{3}H]5-FdUMP following charcoal adsorption of unbound ligand.

surement of the complex of 5-FdUMP with TS and folates in serial tissue biopsies, following treatment with FUra, either alone or in combination with leucovorin.

METHODS

Patients were eligible for the FUra-leucovorin study if they had progressive, evaluable metastatic breast cancer, were able to give signed informed consent, and had WBC$\geq$4000/mm^3 and platelets$\geq$100,000/mm^3, unless myelosuppression was due to marrow involvement by metastatic breast carcinoma. Patients may have had prior chemotherapy.

Treatment Plan.—On days 1 to 5, the patient received leucovorin iv (500 mg/m^2) over 30 minutes. One hour after the termination of the leucovorin infusion, FUra (375 mg/m^2) was given by iv bolus infusion. The cycle was repeated every 21 days if the WBC and platelet counts were adequate; a delay of 7 days was allowed if WBC$\leq$3000/mm^3 or platelets$\leq$75,000/mm^3 on day 1. A 20% reduction in FUra was made for delay in therapy for nadir WBC$<$1000/mm^3, or platelets$<$25,000/mm^3, or for grade II mucositis.

Each patient with accessible tumor received a single cycle of FUra alone at the same dose and schedule described above. The FUra alone was administered as either the first or second cycle for a given patient. The assignment to cycle FUra or FUra-leucovorin alternated; the first patient with accessible tumor who entered on protocol received FUra-leucovorin for the initial cycle, while the second received FUra alone as initial treatment. After the second cycle of treatment, all patients received FUra-leucovorin for all subsequent cycles of treatment.

The patients with accessible skin tumor were biopsied before therapy and 24 hours after the FUra dose (prior to the day 2 dose). A 4-mm punch biopsy of a lesion was taken at each time point.

Biochemical studies.—The skin-punch biopsy was weighed and then homogenized in a glass hand-held homogenizer with KH_2PO_4 (0.2 M, pH 7.2). Half of the cell extract was incubated with 0.1 μM [^{3}H]5-FdUMP, 50 μM 5,10-methylenetetrahydrofolate, in KH_2PO_4 (0.2 M, pH 7.2, with 1% 2-mercaptoethanol) for 30 minutes at 37°C. This measurement was corrected for [6-^{3}H]5-FdUMP exchange with bound "cold" 5-FdUMP during the 30-minute incubation and represents the number of TS binding sites available at the time the biopsy was obtained. The second half of the cell extract was incubated with 0.1 μM [^{3}H]5-FdUMP, 50 μM 5,10-methylenetetrahydrofolate, in NH_4HCO_3 (0.6 M, pH 8.0, with 1% 2-mercaptoethanol) for 3 hours at 37° C, thus allowing complete exchange of all enzyme-bound "cold" FdUMP with the radiolabeled FdUMP. Evidence for completeness of exchange was gleaned through comparisons of total binding sites in control tissue samples obtained prior to FUra treatment with total numbers obtained using the exchange method. The protein-bound [^{3}H]5-FdUMP in this sample represents the total number of TS binding sites available at the time of the biopsy. For both assays, the unbound [^{3}H]5-FdUMP was adsorbed with acid-washed activated charcoal according to published methods (*7*). The charcoal was separated by centrifugation at 10,000$\times g$ for 30 minutes, and the supernatant was counted. All assays were expressed as picomoles 5-FdUMP bound per gram protein.

RESULTS

As this trial has only recently been initiated, this section will include the preliminary results and early follow-up of the initial 13 patients entered on protocol.

Patient Characteristics

At the present time, 13 patients with metastatic breast cancer have been entered on study with a mean follow-up time of 5 months. As table 2 illustrates, the mean age of the patients was 49 years with a range of 34 to 59 years. As this is a phase II study, all patients were heavily pretreated, 92% (12 of 13) having failed prior FUra therapy. All patients failed at least one combination regimen, and 70% had received prior radiation therapy and hormonal therapy. Almost half (5 of 13) had failed other phase I or phase II trials. The sites of disease included skin (39%), bone (69%), liver (31%), lung (15%), and bone marrow (8%).

Toxicity

Toxic side effects from the combination therapy were acceptable and predictable. Myelosuppression was the most common side effect in this heavily pretreated population. Table 3 shows the number of patients who demon-

TABLE 2.—FUra-leucovorin study—patient characteristics

Patients on study	13
Age	49 yr (34–59)
Performance status	78% (40–100%)
Prior therapy	FUra, 12 of 13 (92%)
	Combination chemotherapy, 13 of 13 (100%)
	Hormonal therapy, 9 of 13 (70%)
	Radiation therapy, 9 of 13 (70%)
	Other phase I or II studies, 5 of 13 (40%)

strated either myelosuppression or stomatitis during the course of therapy. Sixty percent of the patients required a 20% dose reduction; the average dose tolerated was 330 mg/m^2/day.

Response to Therapy

Of the 13 patients treated, 4 (31%) responded with a greater than 50% reduction in the size of all measurable disease. A single patient achieved complete remission of all disease, which in this patient was confined to the skin. Responses lasted for 5 to 8 months, all patients subsequently demonstrating progressive disease. Two patients (15%) had progressive disease during the first 2 cycles of therapy, and the 7 remaining patients (54%) have had stable disease after follow-up times of 3 to 8+ months.

TS Binding Studies

Using the techniques as described under the Methods section, serial tissue samples were obtained from 2 patients with accessible skin disease during therapy with FUra alone and with FUra-leucovorin. For each of the 2 cycles, the dose of FUra was identical. The mean amount of TS activity found in the tissue samples was 107 pmol/g protein, and this represented an activity measurement that was on the average 5-fold greater than the background activity inherent in the assay. As table 4 illustrates, the percents of total TS bound by FdUMP at 24 hours following therapy with FUra alone were 0% and 50% for these 2 patients. These same measurements performed at 24 hours following treatment with the combination of FUra and leucovorin demonstrated almost total saturation of the TS enzyme. Both of these patients ultimately responded to therapy with the combination. When patient 1 progressed while on therapy with FUra-leucovorin, similar measurements of TS binding by 5-FdUMP showed no binding of the enzyme by the active metabolite.

DISCUSSION

In this preliminary report of our initial 13 patients treated with the combination of FUra and leucovorin, we found a 31% response rate in a heavily pretreated population of patients with advanced breast cancer. Of these patients, 92% had failed prior therapy with FUra given in combination therapy. Only 2 of 13 patients showed progression of their disease during the first 2 cycles of therapy. The toxicity associated with the combination was acceptable, myelosuppression being dose-limiting. Two of 13 patients had severe myelosuppression during a single cycle of therapy, but this toxicity was obviated by a 20% dose reduction on subsequent cycles. The biochemical studies performed on tissue samples from 2 patients suggest that the addition of leucovorin enhances the saturation of the target enzyme, TS, with FdUMP. These studies further suggest that the prior cellular resistance to FUra alone may have been, in part, due to insufficient intracellular concentrations of folate to effect saturation of the enzyme, which is required for cytotoxicity. This observation is congruent with the reports relating the in vitro FUra sensitivity of cell lines to intracellular folate pool levels (*4–6*).

The degree of binding of 5-FdUMP to the target enzyme in the samples from the 2 patients studied appears to correlate with response. Both patients had failed prior FUra therapy without exogenously administered folate and were able to bind, in their tumor specimens, less than 50% of the available TS in the absence of leucovorin. Complete saturation of the available TS in the presence of exogenously administered leucovorin correlated with a clinically observable response to the combination of FUra-leucovorin. This correlation is further supported by the observation that no binding of FdUMP to TS was detectable in tumor specimens taken from patient 1 during subsequent clinical progression of disease, despite the continued administration of FUra with leucovorin.

Clearly these observations are preliminary and require additional clinical as well as biochemical studies before definitive conclusions are warranted. This trial is ongoing and will be reported in a subsequent manuscript.

TABLE 3.—FUra-leucovorin—toxicity

Toxicity	No./No. of patients	%
Myelosuppression:		
WBC <1,000	1/13	8
1,000–1,900	6/13	46
Platelets <25,000	2/13	15
25,000–50,000	1/13	8
Gastrointestinal:		
Grade 3 stomatitis	1/13	8

TABLE 4.—Percent of tumor TS bound by FdUMP 24 hours following the FUra dose[a]

Patient	FUra alone	FUra + leucovorin
1	52%	98%
2	0%	100%

[a] Each patient was treated with a cycle of FUra plus leucovorin and with FUra alone at an identical dose. Skin biopsies (4 mm) were obtained prior to therapy and 24 hr following the indicated treatment. The samples were processed as per the Methods section, and the amount of bound FdUMP calculated as the percent difference between the total number of TS binding sites and the number of unbound sites in the tumor specimens.

REFERENCES

(*1*) CHABNER BA: Pyrimidine antagonists. *In* Pharmacologic Principles of Cancer Treatment (Chabner BA, ed). Philadelphia: W.B. Saunders Co, 1982, pp 183–212.

(*2*) SANTI DV, MCHENRY CS, SOMMER H: Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. Biochemistry 13:471–481, 1974.

(*3*) DANENBERG PV, LOCKSHIN A: Fluorinated pyrimidines as tight-binding inhibitors of thymidylate synthetase. Pharmacol Ther 13:69, 1981.

(*4*) HOUGHTON JA, SCHMIDT C, HOUGHTON PJ: The effect of derivatives of folic acid on the fluorodeoxyuridylate– thymidylate synthetase covalent complex in human colon xenografts. Eur J Cancer Clin Oncol 18:347–354, 1982.

(*5*) YIN MD, ZAKRZEWSKI SF, HAKALA MT: Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. Mol Pharmacol 23:190–197, 1982.

(*6*) BERGER SH, HAKALA MT: Relationship of dUMP and free FdUMP pools to inhibition of thymidylate synthetase by 5-fluorouracil. Mol Pharmacol 25:303, 1984.

(7) Allegra CJ, Chabner BA, Drake JC, et al: Enhanced inhibition of thymidylate synthetase by methotrexate polyglutamates. J Biol Chem 260:9720–9726, 1985.

(8) Machover D, Schwarzenberg L, Goldschmidt E, et al: Treatment of advanced colorectal and gastric adenocarcinoma with 5-FU combined with high-dose folinic acid. A pilot study. Cancer Treat Rep 66:1803–1807, 1982.

(9) Petrelli N, Madajewicz S, Herrera L, et al: A survival study of 5-fluorouracil and high dose leucovorin in metastatic colorectal carcinoma. Proc ASCO 4:76, 1985.

A Phase II Trial of 5-Fluorouracil and High-Dose Leucovorin in Gastric Carcinoma and a Phase I Trial of Intraperitoneal 5-Fluorouracil and Leucovorin[1]

Susan G. Arbuck,[2,3,]* Harold O. Douglass, Jr.,[2] Fabio Trave,[4,5] and Youcef M. Rustum[3,4,6]

ABSTRACT—Twenty-five patients with advanced measureable gastric carcinoma were treated with D,L-leucovorin (CF) (500 mg/m^2) administered as a 2-hour infusion and FUra (600 mg/m^2) iv push midinfusion. Patients were treated weekly for 6 weeks followed by a 2-week rest. Median age was 57 (range 32 to 82). Median Eastern Cooperative Oncology Group (ECOG) performance status was 2 (range 0 to 4). Thirteen patients had progressed on previous combination chemotherapy that included FUra. At the time of this report, 19 patients were evaluable for response: 3 patients had partial responses, 8 had stable disease, and 8 progressed (but 3 of these received only 3 or fewer treatments before early disease-related death). Two of the responders were previously treated with FUra. Four patients were too early to evaluate. Measureable responses of greater than 50% were seen in bone, liver, lung, and an abdominal mass. Diarrhea occurred in 9 patients and FUra dose reduction was necessary in 8 of them. Other toxicities included lacrimation, rash, nausea, and mucositis. One toxic death occurred. Nine patients with gastrointestinal tumors confined primarily to the intra-abdominal space were treated with ip FUra in escalating doses (2 mM to 4 mM) in combination with D,L-CF in a 2-liter volume, either by 8 consecutive 4-hour dwells (7 patients) or once daily for 5 days (2 patients). The D,L-CF dose was 20.8 μM except for the first day of the 5-day schedule when it was 104 μM. Toxicity included leukopenia, mucositis, nausea and vomiting, skin rash, and abdominal pain. Three episodes of peritonitis resolved with antibiotics. D,L-CF concentration ip (20.8 μM dose) declined from 10.4 ± 3.0 μM at 1 hour to 4.9 ± 2.2 μM at 4 hours, corresponding to a mean absorption half-life of 127 ± 49 minutes and a mean peritoneal clearance of 13.0 ± 4.5 ml/minute. L-CF levels were 3.0 ± 2.6 μM after 4 hours. Some patients with gastric cancer appear to benefit from systemic FUra and D,L-CF, including some with <50% tumor reduction who had improved performance status and/or less dysphagia. Although the response rate to date in this ongoing study is comparable to FUra alone in previously untreated patients, the confidence limits are large. Intraperitoneal FUra and D,L-CF are well tolerated, but further studies are required to determine whether this regimen will be clinically useful.—NCI Monogr 5:203–205, 1987.

Although there has been an unexplained decrease in the incidence of gastric carcinoma in the United States during the past 50 years, this disease remains a major cause of cancer death (*1*). In the United States, most patients present with locally advanced or metastatic disease. Median survival is approximately 6 months despite the use of chemotherapeutic agents that are active in this disease and despite reports of increased response rates with combination chemotherapy. Current survival results are little better than those in a historical series of untreated patients (*2*). Malignant ascites from gastric, pancreatic, and other tumors is another difficult, but not uncommon clinical problem for which currently available therapy is inadequate.

Preclinical studies have demonstrated that 10 μM L-CF concentrations can stabilize the 5-fluorodeoxyuridine monophosphate–thymidylate synthase–reduced folate ternary complex, thereby increasing the cytotoxicity of FUra for some human tumors (*3*). Reports of higher response rates in patients with colon and gastric carcinomas and of responses in patients with carcinoma who had failed previous treatment with FUra (*4–7*) prompted an extension of our clinical studies to patients with gastric carcinoma and also to drug administration by the peritoneal route. This report summarizes the current results of an ongoing phase II trial in gastric carcinoma. A brief description of the phase I trial of intraperitoneal administration, demonstrating its feasibility, and a preliminary report of intraperitoneal CF pharmacokinetics are included.

METHODS

Phase II trial of iv CF and FUra in gastric carcinoma.—Between May 1984 and December 1985, 25 patients with advanced measureable gastric carcinoma entered a phase II trial of intravenous FUra and high-dose CF. Patients were informed of the investigational nature of the treatment and gave written consent according to institutional and federal regulations.

To be eligible, patients had to have an area of known malignant disease measureable by palpation, chest x-ray, liver scan, or computerized axial tomography. Clinical hepatomegaly was followed but not used as an indicator

---

ABBREVIATIONS: CF = leucovorin; 5-CH$_3$-FH$_4$ = 5-methyltetrahydrofolate; ECOG = Eastern Cooperative Oncology Group.

---

[1] Supported by Public Health Service Grants CA-21071, CA-24538, CA-34184, US-Italy Agreement on Cancer Research, and Lederle Laboratories.

[2] Department of Surgical Oncology, Roswell Park Memorial Institute, Buffalo, NY.

[3] Department of Clinical Pharmacology and Therapeutics, Roswell Park Memorial Institute, Buffalo, NY.

[4] Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, NY.

[5] *Present address:* Istituto Nazionale per la Ricerca sul Cancro, Viale Benedetto XV, 10 Genova, Italy.

[6] We thank Mary Baroni, R.N., Suzanne Milliron, R.N., Patricia Goodwin, R.N., Cheryl Frank, and Cheryl Melancon for their assistance.

* *Reprint requests to:* Susan G. Arbuck, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263.

lesion. A life expectancy of at least 2 months was required. Most patients had leukocyte counts ≥4000/mm^3, but later in the study, 2 patients were entered with lower counts since myelosuppression was not dose-limiting. All patients had platelet counts ≥100,000/mm^3.

Patients were treated with D,L-CF (500 mg/m^2) administered as a 2-hour infusion, with FUra (600 mg/m^2) iv push midinfusion. Treatment was administered weekly for 6 weeks of an 8-week cycle. During the 2-week rest that ended each cycle, all patients were reevaluated. Reevaluation was undertaken sooner if there was clinical suspicion of progression.

A complete response was defined as absence of all clinically detectable tumor. A partial response was defined as a reduction of at least 50% of the product of the longest perpendicular diameters of the most clearly measureable tumor mass. No increase in the size of any other lesion and no new areas of malignant disease could appear. There could be no significant deterioration in weight and no deterioration in performance status in patients who were responders. Time to progression is reported from the time treatment was initiated.

Objective progression was defined as a 25% increase in any measureable lesion, the appearance of new areas of malignant disease, or symptomatic deterioration of the performance status by more than one level.

Toxicity is reported using Gastrointestinal Tumor Study Group Criteria.

Phase I trial of ip FUra and CF.—Patients with gastrointestinal tumors confined primarily to the intra-abdominal space were treated with ip FUra in escalating concentrations (2 m*M* to 4 m*M*) in combination with D,L-CF (Lederle Laboratories, Pearl River, NY) in a 2-liter volume of Dianeal containing 1.5% dextrose (Travenol Laboratories, Deerfield, IL), 1,000 U sodium heparin, 8 mEq KCl, and 50 mEq $NaHCO_3$. The schedules were based on those used for ip FUra as a single agent (*8*), but since FUra was used in combination with CF, the starting dose was 2 m*M* (50% of the recommended dose for single-agent ip FUra).

Two treatment schedules were used: 1) Patients whose catheters allowed both inflow and outflow received 8 consecutive 2-liter exchanges, each of 4-hours duration, for a total treatment time of 36 hours, including instillation and drainage. Treatment was repeated every 2 to 3 weeks. The D,L-CF concentration was 20.8 μ*M*. 2) Patients whose catheters allowed inflow but inadequate outflow for the 36-hour schedule received 2 liters daily for 5 days every 28 days. On day 1, the dialysate contained 104 μ*M* D,L-CF; on days 2 to 5 it contained 20.8 μ*M*. Each day it contained 4 m*M* FUra.

TABLE 1.—Phase II trial of iv FUra and CF in gastric carcinoma: Patient characteristics

No. of patients entered	25
Median age in years (range)	57 (32–82)
	No. of patients
Sex	
Males	19
Females	6
ECOG performance status	
0	1
1	7
2	10
3	6
4	1
Previous treatment	
No previous treatment	12
Chemotherapy (including FUra)	13
Radiotherapy	6

TABLE 2.—Phase II trial of iv FUra and CF in gastric carcinoma: Patient status

Evaluable for response	19
Toxic death	1
Too early to evaluate	4
Withdrew (no adverse effects)	1

High-pressure liquid chromatographic analysis of folates in plasma.—Samples for D,L-CF measurement were collected in tubes containing heparin with 1 mg/ml sodium ascorbate. Methotrexate was used as an internal standard. Proteins were precipitated with cold methanol. The supernatant was evaporated and the residue was stored at −20° C until separation and analysis of D,L-CF and 5-methyltetrahydrofolate ($5\text{-}CH_3FH_4$) by reverse-phase, ion-pair high-pressure liquid chromatography. The mobile phase consisted of 30% methanol in 5 m*M* tetrabutylammonium phosphate (Waters Pic-A reagent). D,L-CF and $5\text{-}CH_3FH_4$ were identified on the basis of the retention times of standards injected daily and their 254:294 absorbance ratio. Recovery was approximately 80% and the limit of sensitivity was 0.5 μ*M* for CF and $5\text{-}CH_3FH_4$. A 2112 Redirac Fraction Collector (LKB Products, Bromma, Sweden) was connected to the high-pressure liquid chromatography system for collection of D,L-CF. The biologically active L-CF was measured in peritoneal fluid samples using the microorganism, *Pediococcus cerevisiae.*

RESULTS

Phase II Trial of iv CF and FUra in Gastric Carcinoma

Twenty-five patients have been entered but at the time of this report, not all were evaluable for response. All patients were, however, included in the description of the patient population (table 1) and in the evaluation of toxicity.

Median age was 57 years (range 32 to 82). The population included 19 males and 6 females. The median ECOG performance status was 2 (range 0 to 4). Thirteen patients had progressed on prior chemotherapy that included FUra. Six patients had received previous radiotherapy. Twelve patients had received no previous chemotherapy.

At the time of this report, 19 patients were evaluable for response (table 2). Four patients were too early to evaluate. One patient (who had no adverse effects but who had previous chemotherapy and several surgical procedures) discontinued treatment after 2 weeks. One patient developed fever, grade 3 diarrhea, and myelosuppression, and was admitted to his local hospital with an acute myocardial infarction at week 5 after 4 treatments. Toxicity contributed to his death within 24 hours of hospital admission.

Responses and time to progression are shown in table 3. Three patients had partial responses. Two of the 3 patients had received prior chemotherapy with FUra. Tumor regression was documented in liver, lung, bone, and

Table 3.—Phase II trial of iv FUra and CF in gastric carcinoma: Responses in 20 patients

	No. of patients (%)	Time to progression, weeks
Partial response	3 (15)	15, 25+, 27+
Stable disease	9 (45)	9.5, 12, 12+, 15.5, 20, 20+, 21, 21+, 31+
Progression	8[a] (40)	

[a] The patient who died of toxicity at week 5 before reevaluation is included as a failure. Three patients received 3 or fewer treatments before early disease-related death.

abdominal wall metastases. Nine patients had stable disease, and 5 of these had previously failed treatment that included FUra. Seven patients progressed, but 3 of these received fewer than 4 treatments before early disease-related death. The patient who died of toxicity is included as a failure.

The most frequent toxicity was diarrhea, which occurred in 10 patients. One patient had grade 1, 5 patients had grade 2, and 4 had grade 3 diarrhea. After recovery from diarrhea, all but 1 patient resumed treatment with FUra (500 mg/m^2) without further gastrointestinal toxicity.

Myelosuppression occurred but was less of a clinical problem than diarrhea. Grade 3 leukopenia occurred in 4 patients. Three of these patients had required dose reductions on previous chemotherapy regimens. Leukopenia contributed to the toxic death already described. Although grade 2 thrombocytopenia did occur in 4 patients, it was presumed secondary to other medications in 3 and did not require dose reduction or treatment delay in the fourth patient. Anemia was usually seen in association with bleeding that was tumor- and not treatment-related. Other toxicities included rash in 6 patients, lacrimation in 5, nausea in 3, and mucositis in 2 patients.

Phase I Trial of ip FUra and CF

Seven patients had adenocarcinoma of the pancreas, 1 had adenocarcinoma of the common bile duct, and 1 had hepatocellular carcinoma. Five patients had failed one or more systemic chemotherapy treatments. Median age was 61 (range 41 to 68). Median ECOG performance status was 3 (range 0 to 4). Nine patients were treated with 1 to 4 courses for a total of 16 courses of treatment.

The maximally tolerated FUra dose was 4 mM and toxicity was similar to that previously reported for ip FUra alone (*8,9*). Leukopenia, mucositis, nausea, vomiting, diarrhea, abdominal pain, and skin rash were observed (*10*). Three episodes of peritonitis occurred, but all resolved with antibiotic treatment. No significant clinical responses were seen in this phase I trial.

The D,L-CF concentration ip (20.8 μM dose) declined from 10.4±3.0 μM at 1 hour to 4.9±2.2 μM at 4 hours corresponding to a mean absorption half-life of 127±49 minutes and a mean peritoneal clearance of 13.0±4.5 ml/minutes. L-CF levels were 3.0±2.6 μM at 1 hour. Plasma D,L-CF levels were below the limit of detection.

DISCUSSION

Selected patients with gastric cancer have benefited from systemic FUra and D,L-CF, including some with <50% tumor reduction but with improved performance status, decreased pain, and/or decreased dysphagia. Two of the 3 patients who responded to FUra and D,L-CF had previously received FUra. Longer follow-up and the entry of more previously untreated patients are required in order to estimate the response rate with reasonable confidence limits. The overall response rate to date, however, is comparable to that reported with FUra alone in previously untreated patients. Attempts are being made to develop in vitro assays that could identify those patients who are likely to benefit from FUra modulation by CF.

Intraperitoneal FUra and D,L-CF are well tolerated, but further studies are required to determine whether modulation of ip FUra by CF is a useful approach to pursue clinically. Preliminary results of peritoneal fluid D,L-CF levels in 1 patient who received 104 μM D,L-CF suggest that this dose may be necessary to achieve the desired L-CF concentrations.

REFERENCES

(*1*) Cancer Facts and Figures, 1984. New York: American Cancer Society, 1983.

(*2*) Moertel CE: The natural history of advanced gastric cancer. Surg Gynecol Obstet 126:1071–1074, 1968.

(*3*) Evans RM, Laskin JD, Hakala MT: Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. Cancer Res 41:3288–3295, 1981.

(*4*) Madajewicz S, Petrelli N, Rustum YM, et al: A phase I–II trial of high dose calcium leucovorin and 5-fluorouracil in advanced colorectal carcinoma. Cancer Res 44: 4667–4669, 1984.

(*5*) Machover D, Goldschmidt E, Schwarzenberg L, et al: Treatment of advanced colorectal and gastric adenocarcinomas with 5-fluorouracil combined with high-dose leucovorin: An update. *In* Advances in Cancer Chemotherapy. The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: Park Row, 1984, pp 55–64.

(*6*) Bukowski RM, Cunningham J: Efficacy of 5-fluorouracil in combination with leucovorin in patients with colorectal carcinoma: Continuous infusion of low doses. *In* Advances in Cancer Chemotherapy. The Current Status of 5-Fluorouracil–Leucovorin Calcium Combination (Bruckner HW, Rustum YM, eds). New York: Park Row, 1984, pp 69–72.

(*7*) Bertrand M, Doroshow J, Multhauf D, et al: High dose folinic acid (HDFA) by continuous infusion and iv bolus 5-FU in patients with advanced colorectal cancer: A randomized study. Proc ASCO 4:77, 1985.

(*8*) Speyer JL, Collins JM, Dedrick RL, et al: Phase I and pharmacological studies of 5-fluorouracil administered intraperitoneally. Cancer Res 40:567–572, 1980.

(*9*) Ozols RF, Speyer JE, Jenkins J, et al: Phase II trial of 5-FU administered ip to patients with refractory ovarian cancer. Cancer Treat Rep 68:1229–1232, 1984.

(*10*) Arbuck SG, Trave F, Douglass HO, et al: Phase I and pharmacologic studies of intraperitoneal leucovorin and 5-fluorouracil in patients with advanced cancer. J Clin Oncol 4:1510–1517, 1986.

Transient Acute Hepatotoxicity of High-Dose Methotrexate Therapy During Childhood

Barbara L. Weber,[1,2] Gulten Tanyer,[3] David G. Poplack,[4] Gregory H. Reaman,[5] James H. Feusner,[6] James S. Miser,[4] and W. Archie Bleyer[7,8]*

ABSTRACT—Serial liver-enzyme determinations were performed on 36 children with acute lymphoblastic leukemia who were randomized to receive either conventional intrathecal methotrexate (MTX) therapy with cranial irradiation, or an investigational high-dose MTX regimen consisting of 10 courses of 33,600 mg/m^2 over 24 hours, with high-dose leucovorin rescue. Both groups of patients received intermittent low-dose oral MTX during maintenance therapy. Serum transaminase elevations in the group of conventionally treated patients were infrequent and moderate in severity (<300 IU/liter). In the investigational group, however, the majority of patients had severe, acute increases in SGPT (>300 IU/liter), with peaks up to 1000 to 2000 IU/liter. The incidence and severity of acute transaminasemia were directly proportional to the number of high-dose MTX courses received: courses 1, 2, 3, 4, 5, and 6 caused transaminase elevations in 31%, 50%, 50%, 73%, 100%, and 100% of courses, respectively, and 0%, 14%, 20%, 44%, 55%, and 92%, respectively, were over 300 IU/liter. Patients in both treatment groups developed a pattern of increasing serum alkaline phosphatase concentrations after initiation of low-dose oral MTX therapy; isoenzyme analysis indicated that this effect was osseous rather than hepatic. Serum bilirubin was rarely elevated. Transaminases returned to normal within 1 to 2 weeks after each high-dose MTX treatment, and with follow-up for as long as 7 years, no patient has developed clinical evidence of residual liver disease, after 3 years of high-dose MTX therapy and multiple other antileukemia drugs. The acute hypertransaminasemia frequently observed after high-dose MTX therapy is transient and reversible, and, in children, does not appear to result in chronic liver disease.—NCI Monogr 5:207–212, 1987.

Shortly after folate antagonists were found useful in the treatment of acute leukemia, they were recognized to be toxic to normal liver tissue (*1*). Several reports on the nature of MTX hepatotoxicity followed, but many aspects of this adverse drug reaction remained undefined. The dependence of incidence and severity on the dose schedule and on the route of administration, for example, is incompletely characterized. It was apparent that MTX-induced cirrhosis was a risk in adults with psoriasis treated with low doses daily (*2,3*), and that intermittent high-dose MTX produced, in some patients, hepatic dysfunction (*4,5*).

In our study, children with acute lymphoblastic leukemia were treated with MTX in doses approximately 3 times greater than the highest doses previously reported. This study of hepatic dysfunction after high-dose MTX therapy was undertaken after our initial clinical trial demonstrated serum liver-enzyme levels following MTX that were higher than previously reported. A prior publication had reported that infusions of high-dose MTX caused severe hepatotoxicity [peak SGOT (aspartate aminotransferase) greater than 150 IU/liter] (*5*). In contrast, after 6 courses of high-dose MTX therapy, 90% of our patients had SGPT (alanine aminotransferase) levels greater than 300 IU/liter and peak levels greater than 1500 IU/liter were not uncommon. Serum transaminase levels did return to normal in all of our cases, and clinical evidence of residual liver disease has not been observed in any patient, with follow-up for up to 7 years. We present these observations in support of continuing therapeutic trials of high-dose antifolate therapy in children and we offer suggestions for long-term evaluation.

PATIENTS AND METHODS

Between 1979 and 1981, 36 children at the Children's Hospital and Medical Center in Seattle, Washington, entered the toxicity study reported here. The patients had average- or high-risk acute lymphoblastic leukemia according to criteria of the Children's Cancer Study Group (age at diagnosis, less than 3 or more than 6 years; WBC greater than 10,000/mm^3). They were treated according to a protocol designed to compare conventional CNS prophylaxis with cranial irradiation and intrathecal MTX (regimen 1) to an alternative investigational CNS therapy with very high-dose MTX in conjunction with high-dose leucovorin rescue (regimen 2). The patients ranged in age at diagnosis from 8 months to 17 years; 19 were male. A 2:1 weighted randomization between these two forms of CNS therapy resulted in 24 of 36 patients being treated according to regimen 2. Three patients had CNS leukemia at diagnosis and were nonrandomly assigned to regimen 2.

Induction therapy.—Patients were induced into remission with vincristine, L-asparaginase, prednisone, and daunomycin. In addition, patients on regimen 1 received 1 6- to 12-mg dose of intrathecal MTX, depending on age. Instead of intrathecal MTX, patients on regimen 2 received 1 course of high-dose MTX and high-dose leucovorin rescue therapy according to the following schedule. A priming

---

ABBREVIATION: MTX = methotrexate.

[1] University of Washington School of Medicine, Seattle, WA.

[2] *Present address:* Dana-Farber Cancer Institute, Boston, MA.

[3] University Medical School, Samsun, Turkey.

[4] Pediatric Branch, National Cancer Institute, Bethesda, MD.

[5] Children's Hospital National Medical Center, Washington, DC.

[6] Children's Hospital and Medical Center of Northern California, Oakland, CA.

[7] Children's Orthopedic Hospital and Medical Center, Seattle, WA.

[8] American Cancer Society Professor of Clinical Oncology.

* *Reprint requests to:* W. Archie Bleyer, M.D., P.O. Box C-5371, Seattle, WA 98105.

dose of MTX, 6,000 mg/m^2, was administered over 1 hour, followed immediately by a constant infusion of MTX, 1200 mg/m^2/hour for 23 hours. The total dose per course of therapy was 33,600 mg/m^2 over 24 hours. Twelve hours after completion of the MTX infusion (36 hours from the start), 200 mg/m^2 leucovorin was administered iv over 1 hour. Three hours later, leucovorin was started at doses of 12 mg/m^2 iv every 3 hours for 5 doses, then every 6 hours until the serum MTX level fell below 1×10^{-7} *M*. Throughout the periods of MTX and leucovorin therapy, urine pH was maintained above 6.5 with sodium bicarbonate in the iv fluids. Diuresis was maintained with iv fluids at 3,000 ml/m^2/day.

Consolidation therapy.—Systemic therapy for all patients during the consolidation phase consisted of vincristine, prednisone, and 6-mercaptopurine. Regimen 1 patients also received 4 additional weekly injections of intrathecal MTX in doses described above, together with cranial irradiation in age-dependent doses ranging from 1800 to 2400 rad in 200-rad fractions. In place of intrathecal MTX and cranial irradiation, regimen 2 patients received 3 courses of very high-dose MTX and high-dose leucovorin therapy as described above at 2-week intervals. If the patient could be discharged on oral fluids and medications, leucovorin was often given orally for the last few days if the plasma MTX level fell below 10^{-6} *M* and continued until it fell below 10^{-7} *M*. Each dose of oral leucovorin was taken with a tablet of acetazolamide, 125–250 mg depending on age, in an attempt to maintain urinary alkalinization.

Maintenance therapy.—Six 23-week cycles of prednisone, vincristine, 6-mercaptopurine, L-asparaginase, cyclophosphamide, daunomycin, and twice-weekly oral MTX (7.5 mg/m^2 during weeks 3 to 6, 10 to 13, and 17 to 20) were administered to all patients. Regimen 1 patients also received oral MTX (7.5 mg/m^2) during the first 5 days of every maintenance cycle. Regimen 2 patients received a course of high-dose MTX and leucovorin therapy, as described in the Consolidation Therapy section above, at the beginning of each maintenance cycle. Regimen 2 patients also received high-dose 6-mercaptopurine (500 mg/m^2/day) on days 1 through 5 of each maintenance cycle. All therapy was discontinued after 6 maintenance cycles (approximately 3 years).

All patients had liver-enzyme levels determined at regular intervals throughout therapy. Upper limits of normal values for our laboratory were: SGPT, 40 IU/liter; total bilirubin, 0.8 mg/dl; direct bilirubin, 0.3 mg/dl; alkaline phosphatase, 180 IU/liter for patients between 1 year of age and adolescence, 260 IU/liter for adolescent females, and 350 IU/liter for adolescent males. An evaluable cycle was defined as a course of MTX therapy (intrathecal or iv) with at least 1 of the above levels determined both immediately before and within 14 days after administration of MTX. A cycle was considered normal if liver-enzyme levels were normal both before and after therapy. A cycle was considered abnormal if liver-enzyme levels were normal before and abnormal after a course of MTX. "Other" cycles are those in which abnormal liver-enzyme levels were observed both before and after therapy.

Baseline SGPT levels were defined as the last values determined before the next course of therapy and at least 14 days after the last course of MTX. Peak SGPT levels were defined as the highest level in the 4 days immediately following MTX administration. Baseline alkaline phosphatase levels were defined in the same manner as baseline SGPT levels, i.e., the last value obtained prior to the next cycle of therapy and at least 14 days after the last course of MTX. In order to correct for the effect of age on normal values, peak alkaline phosphatase levels were expressed as the maximum change in IU/liter that occurred in the 4 days immediately following MTX administration.

One patient treated with high-dose MTX on regimen 2 developed pancreatitis and jaundice shortly after beginning the first maintenance cycle. This episode was attributed to L-asparaginase toxicity. The patient's direct bilirubin levels peaked at 9.5 mg/dl and returned to normal within 5 weeks. Eight weeks after direct bilirubin levels returned to normal, SGPT levels began to rise and fluctuated between 2 and 5 times normal for the next 7 months. A diagnosis of chronic active hepatitis was made when Epstein-Barr viral capsid antigen titers proved consistent with persistent infection. It is unlikely that these transaminase elevations occurred as a result of MTX therapy, in that the time course and clinical presentation did not resemble previously reported episodes of MTX-induced liver damage. Moreover, subsequent maintenance courses in this patient with high-dose MTX did not reactivate either pancreatic or hepatic disease. Data from this maintenance cycle were therefore not included in the analysis below.

RESULTS

The 2 groups of patients on regimens 1 and 2 appeared to be comparable with respect to risk factors for the development of liver disease. No patient had a pretreatment history of exposure to hepatotoxic agents, episodes of jaundice, acholic stools or dark urine, or specific complaints of right upper quadrant tenderness. Hepatomegaly at diagnosis, presumed due to leukemic infiltration, was equally common in both groups, and during therapy no patient developed ascites, hepatosplenomegaly, or hepatic tenderness.

Abnormal Cycles

Regimen 1

Eighteen cycles were considered evaluable, with liver-enzyme levels obtained both before and within 14 days after intrathecal MTX was administered. Fourteen of 18 cycles (78%) were normal, with either no change in enzyme levels after treatment or changes within the normal range. Four of 18 (22%) were abnormal. All abnormal cycles occurred during induction. In all cases, the peak elevation of liver-enzyme levels was less than twice normal (<120 IU/liter).

Regimen 2

Seventy-five cycles were considered evaluable. The trend of increasing numbers of abnormal cycles throughout the course of treatment is illustrated in figure 1. During induction, 69% of cycles were normal, while 100% of maintenance cycles eventually became abnormal. Other cycles occurred in a small number of patients and only during the end of the consolidation phase.

SGPT

Regimen 1

Baseline SGPT levels were available in 27 of 47 cycles. Throughout induction and consolidation, all 15 measured levels were within the normal range. During maintenance

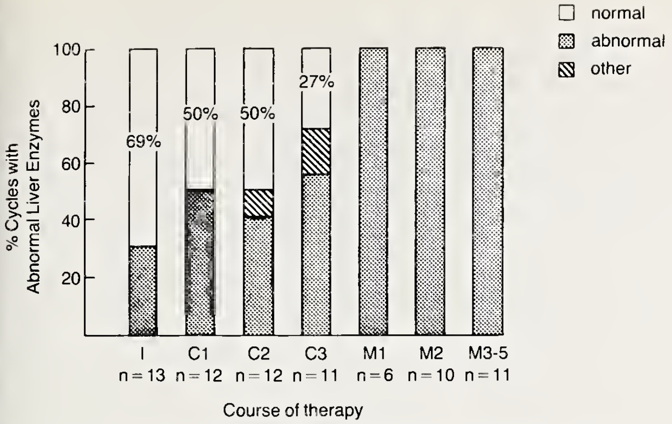


FIGURE 1.—Percent of cycles with abnormal liver enzymes as a function of course of therapy for patients on regimen 2. I = Induction; C = Consolidation; M = Maintenance.

therapy, 9 of 12 were within the normal range and 3 of 12 were elevated less than twice normal. Peak SGPT levels were available for 5 courses of intrathecal MTX. Four of 5 levels were within the normal range; 1 level determined during induction was slightly elevated.

Regimen 2

Baseline SGPT levels were available in 99 of 148 cycles (table 1). Fifteen percent of patients had slightly elevated SGPT levels during induction, and 37% had mildly elevated baseline values before the second or third consolidation course of high-dose MTX therapy. All patients had normal baseline SGPT levels before beginning the first maintenance cycle.

Peak SGPT levels were available in 59 cycles. The trend for increasing incidence and severity of abnormal peak SGPT levels is illustrated in figure 2. During induction therapy, 9 of 13 patients (69%) had SGPT levels within the normal range and no patient experienced severe elevations. During consolidation and maintenance therapy, the number of courses with peak levels in the normal range fell from 12 for the first 24 consolidation courses to none of 27 maintenance courses. Concurrently, the number of patients experiencing severe elevations rose from 1 of 8 after the first consolidation course to 10 of 11 (92%) after 3 maintenance cycles.

Alkaline Phosphatase

Regimen 1

Baseline alkaline phosphatase levels were available in 32 cycles. Figure 3 illustrates the trend for increasing baseline levels throughout the course of treatment. Isoenzyme determinations performed in 3 patients with elevated levels demonstrated that in each case the elevation was due to the isoenzyme derived from bone. Peak alkaline phosphatase levels were available in 4 cycles. All were within the normal range, and maximum change in alkaline phosphatase levels ranged from a decrease of 11 IU/liter to an increase of 47 IU/liter.

Regimen 2

Baseline alkaline phosphatase levels were available in 76 cycles and followed the same trend as those determined in regimen 1 patients (fig. 3). Combined mean levels for regimen 1 and regimen 2 patients rose from 107 IU/liter after induction to the upper limit of normal at 181 IU/liter after the first maintenance cycle. After 3 maintenance cycles, the mean was well above the normal range at 251 IU/liter. Isoenzyme determinations in 6 patients with increased levels again indicated that in all instances the osseous component was primarily responsible for the elevation in the total amount of serum alkaline phosphatase. Peak alkaline phosphatase levels were available in 53 cycles. Because serum alkaline phosphatase levels are age- and sex-dependent, and because the baseline enzyme values declined, then rose during therapy (fig. 3), absolute values for alkaline phosphatase peak levels were difficult to evaluate. Figure 4 suggests a trend of increasing maximum change throughout treatment. The mean value during induction decreased 25 IU/liter and subsequently increased 25 IU/liter during late consolidation and increased 45 IU/liter after 3 or more maintenance cycles. In general the peak in alkaline phosphatase occurred 1 to 2 days after, and at a lower value relative to the peak in SGPT.

TABLE 1.—Baseline SGPT levels prior to courses of therapy (regimen 2)

Course of therapy	No. of patients	Pre-MTX SGPT, IU/liter		
		<60	60–600	>600
Preinduction	16	94%	6%	0
Induction	20	85%	15%	0
Consolidation 1	16	63%	37%	0
Consolidation 2	15	87%	13%	0
Consolidation 3	7	100%	0	0
Maintenance 1	18	100%	0	0
Maintenance 2	13	100%	0	0
Maintenance 3–4	10	100%	0	0

Bilirubin

As illustrated by the patient mentioned in the Patients and Methods section above, elevations in direct serum bilirubin concentration have been observed in patients on the high-dose MTX regimen, but these have been less common than either SGPT or alkaline phosphatase elevations. The peak values tended to occur later than those observed with

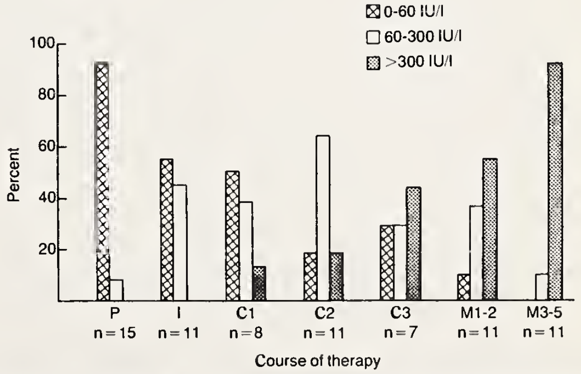


FIGURE 2.—Percent of cycles with peak SGPT levels falling within the given ranges as a function of course of therapy for patients on regimen 2. P = Preinduction; I = Induction; C = Consolidation; M = Maintenance.

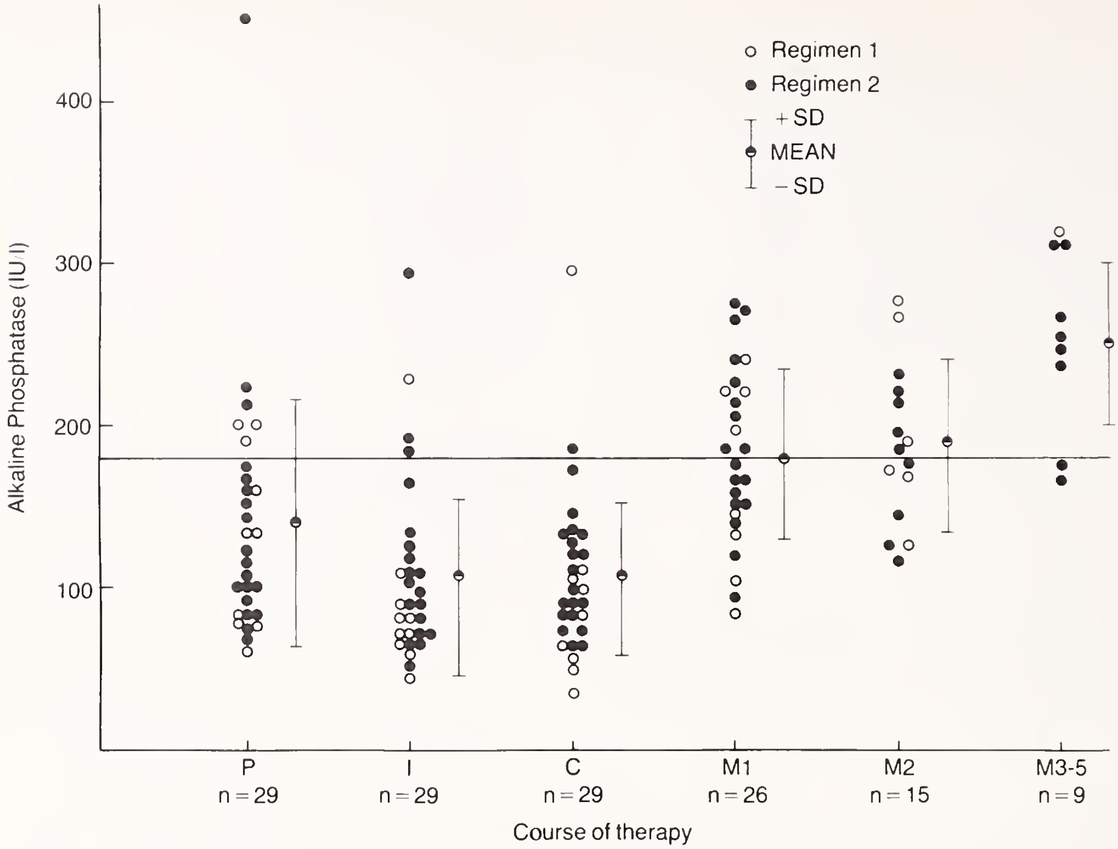


FIGURE 3.—Baseline alkaline phosphatase levels for patients on regimen 1 and regimen 2 as a function of course of therapy. *Vertical bars* indicate mean and standard deviation values of patients on both regimens. P = Preinduction; I = Induction; C = Consolidation; M = Maintenance.

the enzymes, usually 2 to 3 days after the peak in SGPT. Also, the peaks were proportionately lower relative to pretreatment values than the relative increments observed with either of the enzyme changes.

Late Effects Evaluation

Two patients who experienced hematologic relapse and 3 who suffered CNS relapse were removed from regimen 2. The 19 remaining patients completed 3 years of therapy, including 10 courses of high-dose MTX therapy, and had no evidence of residual liver dysfunction at the time of their last follow-up 4 to 7 years after starting therapy and 1 to 4 years after completing therapy. No patient has had symptoms of hepatic abnormalities. SGPT, alkaline phosphatase, and bilirubin values have been normal, and physical examinations have revealed no evidence for hepatic tenderness, hepatomegaly, splenomegaly, jaundice, telangiectasia, or varices.

DISCUSSION

Shortly after folic acid antagonists were found to be useful in the treatment of acute leukemia, their possible role in the development of cirrhosis was questioned (*1*). Studies demonstrating an increased incidence of cirrhosis in adults treated with MTX for psoriasis added to the concern that as improved therapy for acute lymphoblastic leukemia increased the number of long-term survivors, some patients would develop end-stage liver disease and die as a result of therapy rather than leukemia. All recent studies have reported a varying incidence and severity of portal fibrosis, inflammation, and steatosis (*1,4-10*). However, only 2 groups have reported histologically proven cirrhosis (*6,7*). Reports indicating that hepatic fibrosis is not progressive after discontinuing therapy, and in some cases appears to regress, are reassuring (*8,9*).

The results of our study show an increasing incidence and severity of acute transaminasemia as a function of the number of high-dose MTX infusions administered. This trend was not observed in control patients receiving conventional oral and intrathecal MTX therapy. Additionally, we noted rising pretreatment alkaline phosphatase levels, which appear to be derived from bone rather than liver in patients on both regimens after maintenance therapy is initiated. Moderate increases in serum alkaline phosphatase, due primarily to the osseous isoenzyme, were observed during the 4 days after MTX therapy. Finally and most significantly, none of the 19 children in this study who were treated with 10 courses of high-dose MTX during a 3-year period and evaluated 1 to 4 years after completing this therapy had clinical evidence of residual liver disease.

In addition, when all patients in the NCI 77-02/CCG-191 study are included, whether or not they were part of this in-depth study of acute hepatic toxicity, a difference in residual hepatic function between the groups of children treated with high- and standard-dose MTX has not been apparent. In September 1985, 117 patients were in complete remission and 77 of these patients had completed 3 years of protocol-specified therapy and were in the off-treatment observation phase. Fifty patients on the high-dose MTX regimen and 27 on the conventional therapy regimen were off treatment. No clinical evidence for residual hepatic dysfunction had been documented in any of the patients. This comparison indicates that the very high doses of MTX used in the investigational regimen obviously does not increase the long-term hepatic toxicity of antimetabolite therapy in children with leukemia.

Acute changes in transaminase levels are dose-related. Serum MTX levels after intrathecal MTX are much lower and reach a peak more slowly than serum levels after a very high dose of MTX administered iv. Marked transaminase elevations have not been noted after intrathecal MTX administration. On the other hand, the rapid rise and high peak levels of MTX in the serum of patients treated with very high-dose iv MTX (regimen 2) was repeatedly and predictably associated with acute increases in transaminase levels. In most patients, the elevated levels returned rapidly to the normal range, possibly reflecting a transmembrane efflux of enzymes from intact hepatocytes during the period of maximum MTX concentration rather than hepatocellular necrosis.

The only cycles in which transaminase levels did not return to normal before the next course of therapy were those at the end of the consolidation phase of therapy. During consolidation therapy, high-dose MTX was administered every other week for 6 weeks, whereas during maintenance therapy, the high-dose MTX was given once every 23 weeks. Hence the pattern of rising pretreatment serum transaminases is probably a result of the cumulative effect of repeated, frequent high-dose MTX exposure. Cumulative damage is known to be associated with fibrosis, steatosis, and inflammation. However, no studies reported to date have demonstrated biopsy findings consistent with cirrhosis in patients with normal pretreatment transaminase levels and without clinical evidence of liver disease.

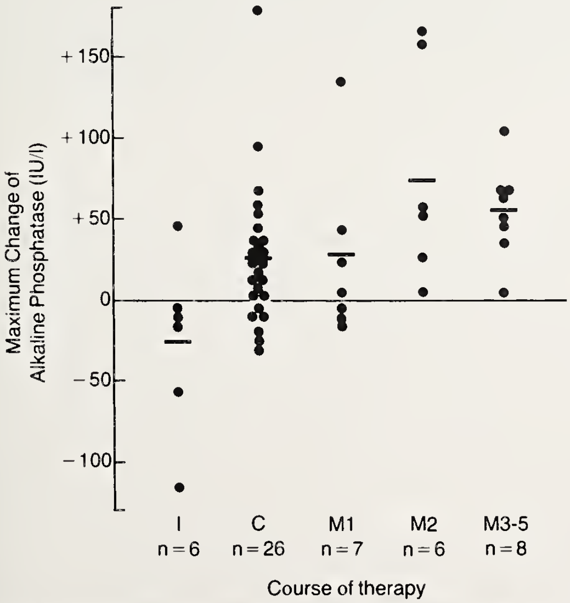


FIGURE 4.—Maximum change in alkaline phosphatase levels after high-dose MTX therapy in patients on regimen 2. The change represents the difference between the pretreatment value and the highest level during the 4-day period after MTX therapy. I = Induction; C = Consolidation; M = Maintenance.

Our patients have also shown a progressive rise in their pretreatment skeletal alkaline phosphatase levels during maintenance therapy. This trend was noted in patients receiving very high-dose MTX and low-dose oral MTX (regimen 2), as well as those receiving oral MTX only (regimen 1). This observation suggests that alkaline phosphatase elevation is a result of low-dose oral therapy or other components of therapy, or is in some way related to the disease process itself. A few studies have reported toxic effects of MTX on the skeletal system (*6,11*), and preliminary results indicate that the major alkaline phosphatase isoenzyme elevated in our patients after initiation of maintenance therapy is derived from bone and not from liver. This finding further supports our conclusions that residual liver damage is not a clinically significant problem despite the marked, acute liver-enzyme abnormalities. A study is currently under way to further define bone toxicities in these patients.

What is needed to assess the long-term effects of MTX therapy on the liver are tests which measure actual liver function. Two such tests which have been shown to correlate well with biopsy findings are indocyanine green clearance, which is dependent on hepatic blood flow, and [^{14}C]aminopyrine metabolism, a measure of hepatic microsomal enzyme function (*12*). Both are noninvasive and relatively easy to administer and have been used in assessment of long-term MTX effects (*13*).

Liver biopsy remains the only definitive method of assessing histologic changes. Biopsies should be considered in certain patients. First, they may be indicated in patients with persistent clinical evidence of liver disease such as ascites, hepatomegaly with tenderness, or varices. Second, patients in whom elevated serum transaminase levels fail to return to normal between courses of therapy could be considered for biopsy before additional high-dose MTX therapy is administered. Patients with evidence of abnormal liver function on more specific hepatic function testing, such as indocyanine green clearance or aminopyrine metabolism, may be candidates for biopsy. Finally, a strong clinical suspicion of viral hepatitis may be indication for biopsy. In 1 recent patient of ours, for example, the serologic studies for hepatitis B antigen and antibody were repeatedly negative, but an open-liver biopsy demonstrated the core antigen within hepatocytes.

In summary, we are reassured by the finding of normal transaminase levels between courses of therapy. The clinical hepatotoxicity of MTX administered to children with acute leukemia according to the treatment protocol described in this report is not severe enough to warrant discontinuing MTX therapy. We have not as yet defined a need for routine serial liver biopsies in these patients. Quantitative measurement of liver function and possibly liver biopsy may be indicated, however, in patients with a delayed recovery from hypertransaminasemia or any evidence of clinical hepatic failure.

REFERENCES

(*1*) COLSKY J, GREENSPAN EM, WARREN TN: Hepatic fibrosis in children with acute leukemia after therapy with folic acid antagonists. Arch Pathol Lab Med 59:198–206, 1955.

(*2*) Podurgiel BJ, McGill DB, Ludwig J, et al: Liver injury associated with MTX therapy for psoriasis. Mayo Clin Proc 48:787–792, 1973.

(*3*) Weinstein GD: Methotrexate. Ann Intern Med 86:199–204, 1977.

(*4*) McIntosh S, Davidson KL, O'Brien RT, et al: Methotrexate hepatotoxicity in children with leukemia. J Pediatr 90:1019–1021, 1977.

(*5*) Perez C, Sutow WW, Wang YM, et al: Evaluation of overall toxicity of high dose MTX regimens. Med Pediatr Oncol 6:219–228, 1979.

(*6*) Armitage JO, Burns CP, Kent TH: Liver disease complicating the management of acute leukemia during remission. Cancer 41:737–742, 1978.

(*7*) Nesbit M, Krivit W, Heyn R, et al: Acute and chronic effects of MTX on hepatic, pulmonary and skeletal systems. Cancer 37:1048–1054, 1976.

(*8*) Parker D, Bate CM, Craft AW, et al: Liver damage in children with acute leukemia and non-Hodgkin's lymphoma on oral maintenance chemotherapy. Cancer Chemother Pharmacol 4:121–127, 1980.

(*9*) Topley JM, Benson J, Squier MV, et al: Hepatoxicity in the treatment of acute lymphoblastic leukemia. Med Pediatr Oncol 7:393–399, 1979.

(*10*) Roenigk HH, Bergfeld WF, St. Jacques R, et al: Hepatotoxicity of MTX in the treatment of psoriasis. Arch Dermatol 103:250–261, 1971.

(*11*) Ragab AM, Fiech RS, Vieth TS: Osteoporotic fractures secondary to methotrexate therapy of acute leukemia in remission. Cancer 25:580–558, 1970.

(*12*) Carlisle R, Galambos JT, Warren WD: The relationship between conventional liver tests, quantitative function tests and histopathology in cirrhosis. Dig Dis Sci 24:385–392, 1979.

(*13*) Birnie GG, Fitzsimons CP, Czarnecki D, et al: Hepatic metabolic function in patients receiving long-term methotrexate therapy: Comparison with topically treated psoriatics, patient controls and cirrhotics. Hepatogastroenterology 32:163–167, 1985.

Recent Preclinical and Clinical Studies With the Thymidylate Synthase Inhibitor N^{10}-Propargyl-5,8-dideazafolic Acid (CB 3717)[1]

A. Hilary Calvert,[2,*] David R. Newell,[2] Ann L. Jackman,[2] Lindsey A. Gumbrell,[2] Ewa Sikora,[2,3,4] Barbara Grzelakowska-Sztabert,[2,3] Joel A. M. Bishop,[2] Ian R. Judson,[2] Stephen J. Harland,[2] and Kenneth R. Harrap[2,5]

ABSTRACT—CB 3717, N^{10}-propargyl-5,8-dideazafolic acid, is a tight-binding inhibitor of thymidylate synthase (TS) whose cytotoxicity is mediated solely through the inhibition of this enzyme. Recent preclinical studies have focused on the intracellular formation of CB 3717 polyglutamates. Following a 12-hour exposure of L1210 cells to 50 μM [^{3}H]CB 3717, 30% of the extractable radioactivity could be accounted for as CB 3717 tetra- and pentaglutamate, as determined by high-pressure liquid chromatography (HPLC) analyses. As inhibitors of isolated L1210 TS, CB 3717 di-, tri-, tetra- and pentaglutamate are 26-, 87-, 119- and 114-fold more potent than CB 3717, respectively, and their formation may, therefore, be an important determinant of CB 3717 cytotoxicity. In early clinical studies with CB 3717, activity has been seen in breast cancer, ovarian cancer, hepatoma, and mesothelioma. Toxicities included hepatotoxicity, malaise, and dose-limiting nephrotoxicity. This latter effect is thought to be due to drug precipitation within the renal tubule as a result of the poor solubility of CB 3717 under acidic conditions. In an attempt to overcome this problem, a clinical trial of CB 3717 administered with alkaline diuresis is under way. Preliminary results at 400 and 500 mg/m^2 suggest that a reduction in nephrotoxicity may have been achieved with only 1 instance of renal toxicity in 10 patients. Hepatotoxicity and malaise are again the most frequent side effects. Evidence of antitumor activity has been seen in 3 patients. Pharmacokinetic investigations have shown that alkaline diuresis does not alter CB 3717 plasma levels or urinary excretion and that satisfactory urinary alkalinization can be readily achieved.—NCI Monogr 5:213–218, 1987.

The synthesis of CB 3717, N^{10}-propargyl-5,8-dideazafolic acid (fig. 1) and its introduction into clinical practice represent a major departure from established antifolate cancer chemotherapy (*1,2*). Unlike traditional antifolates, the cytotoxicity of CB 3717 is not mediated by the inhibition of dihydrofolate reductase (DHFR). Evidence from a number of studies strongly implicates TS, the terminal enzyme in the de novo pathway for thymidine monophosphate biosynthesis, as the sole locus for the cytotoxic action of CB 3717. Thus, CB 3717 cytotoxicity in vitro and antitumor activity in vivo can be reversed by thymidine alone (*1,3,4*). At cytotoxic concentrations, CB 3717 causes a reduction in intracellular thymine nucleotide pools, an elevation of deoxyuridine monophosphate levels and no elevation in dihydrofolate concentrations (*3*). In vivo, CB 3717 produces an elevation of plasma deoxyuridine and a depletion of thymidine (*4*). Cells resistant to methotrexate (MTX) due to raised DHFR levels are not cross-resistant to CB 3717 cytotoxicity (*1,5*). Finally, L1210 cells with acquired resistance to CB 3717 overproduce TS 45-fold and DHFR only 2-fold (*6*). Against isolated rodent (L1210) and human (W1–L2) TS, CB 3717 is a tight-binding competitive inhibitor with an inhibitory constant (Ki) of 4 nM (*3,7*). As such, it is, among monoglutamates, the most potent folate-based TS inhibitor described to date.

Although CB 3717 displays appreciable in vivo antitumor activity in only one experimental antitumor system (*1*), the potent and selective biochemical properties of this molecule were a powerful stimulus leading to its selection for clinical trial in 1981. In preclinical toxicity studies in mice, the dose-limiting toxicity was found to be renal (Calvert AH, Jackman AL, unpublished results). Subsequently, hepatotoxicity has been demonstrated in mice and rats (*8*). Associated pharmacokinetic studies in rodents have shown that biliary excretion is the major route of elimination at nonhepatotoxic doses (*8*) and that there is accumulation and retention of the drug in the kidneys (*9*). The mechanism underlying this latter observation is thought to be drug precipitation within the renal tubule as a result of the poor solubility of CB 3717 under acid conditions. This effect presumably causes the nephrotoxicity of CB 3717.

In early clinical trials (*2*), the dose-limiting toxicity of CB 3717, given by a 1-hour infusion, was found to be renal, occurring at doses of >400 mg/m^2. However, dose-unrelated hepatotoxicity and malaise were the most frequent side effects encountered, with an incidence of >50%. During these early studies, evidence of antitumor activity was observed in patients with ovarian cancer, breast cancer,

---

ABBREVIATIONS: DHFR = dihydrofolate reductase; HPLC = high-performance liquid chromatography; K_i = inhibitory constant; $K_{i,app}$ = apparent inhibitory constant; MTX = methotrexate; TS = thymidylate synthase; WHO = World Health Organization.

---

[1] This work was supported by grants from the Medical Research Council (UK) and Cancer Research Campaign (UK).

[2] Sections of Drug Development and Medicine, Institute of Cancer Research, Sutton, Surrey, UK.

[3] Visiting workers from The Nencki Institute of Experimental Biology, Warsaw, Poland.

[4] Recipient of a CRC International Fellowship awarded by the International Union Against Cancer.

[5] We thank ICI Pharmaceuticals Plc for providing the material for clinical studies and for their permission to quote unpublished data; Professors A. L. Harris and S. B. Kaye for permission to cite their clinical results with CB 3717; Dr. K. Pawelczak for providing samples of CB 3717 polyglutamates; and Miss Ann Robinson for preparation of this manuscript.

* *Reprint requests to:* Dr. A. H. Calvert, Drug Development Section, Institute of Cancer Research, Block E, Clifton Avenue, Belmont, Sutton, Surrey SM2 5PX, England.

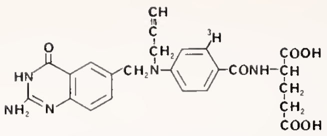


FIGURE 1.—Structure of CB 3717. ^{3}H indicates the position of the tritium label in [^{3}H]CB 3717.

and mesothelioma. This in turn stimulated the phase II evaluation of CB 3717.

In phase II trials of CB 3717, 400 mg/m^2 given every 3 weeks, conducted by the authors and Professors A. L. Harris (Newcastle, UK) and S. B. Kaye (Glasgow, UK), activity in ovarian cancer, breast cancer, hepatoma, and mesothelioma has been demonstrated [(*10,11*); unpublished results]. No activity was found in colorectal cancer.

In view of these early clinical results with CB 3717, further preclinical and clinical studies are in progress. Current preclinical investigations are aimed at defining the extent and importance of CB 3717 polyglutamation. Studies from other groups have shown that CB 3717 is a substrate for folylpolyglutamate synthetase (*12*) and that the polyglutamated derivatives are markedly more potent inhibitors of TS than is CB 3717 (*13*). Evidence from in vivo studies suggests that CB 3717 is converted to polyglutamated forms in mouse liver and kidney (*14*). In the present study, we have sought to demonstrate the formation of these metabolites in tumor cells grown in vitro and to characterize their inhibition of mammalian TS.

Current clinical work with CB 3717 is focusing on measures to prevent the dose-limiting nephrotoxicity, thereby allowing further dose escalations. Experiments to determine the solubility of CB 3717 have shown that it is both pH- and NaCl-concentration-dependent (Hughes L, personal communication). For example, in a sample of human urine, the solubility of CB 3717 was found to be 0.04, 0.4, and 4.0 mg/ml at pH 7, 8, and 9, respectively (Newell DR, unpublished results). In an ongoing clinical study, alkaline diuresis has been employed in an attempt to prevent CB 3717 nephrotoxicity. These studies and associated pharmacokinetic investigations are reported herein.

PRECLINICAL STUDIES

Materials and Methods

Preparation and purification of [^{3}H]-CB 3717.—[Benzoyl-^{3}H]CB 3717 diethyl ester was provided as an ethanolic solution at a radioactive concentration of 2 mCi/ml and a specific activity of 10.5 Ci/mmol (Amersham Plc, UK, custom synthesis). Preparation of CB 3717 was performed as required by the following method. The required volume of the ethanolic solution was evaporated to dryness with N_2 at 30°–40°C, reconstituted in 200 μl 0.1 *M* NaOH and allowed to stand at room temperature for 4 hours. The solution of [^{3}H]CB 3717 was neutralized with acetic acid and then purified by HPLC on a 15×0.46 cm μBondapak C18 column (Waters Associates, Harrow, UK), eluted at 2 ml/minute with 10% CH_3CN/90% 0.1 *M* $NaCH_3COO$ pH 7 (vol/vol). The fractions corresponding to [^{3}H]CB 3717 were collected and pooled, and nonradioactive CB 3717 added to give a final specific activity of 0.15 Ci/mmol. This solution was added directly to the cultures of L1210 cells to give a final CH_3CN concentration of <1%, which did not affect cell growth.

CB 3717 polyglutamate formation.—CB 3717 di-, tri-, tetra-, and pentaglutamate (the prefixes represent total glutamates) were synthesized by Dr. K. Pawelczak (Opole, Poland). In experiments to determine the formation of CB 3717 polyglutamates in L1210 cells, cells suspended at 2×10^5/ml were exposed to 50 μM [^{3}H]CB 3717 (0.15 Ci/mmol) in RPMI 1640 medium containing 10% donor horse serum, 2 m*M* L-glutamine and 50 μM thymidine for 12 hours at 37°C. Thymidine was present to prevent cell death during the experiment. Following incubation, cells were washed twice with phosphate-buffered saline at 4°C, resuspended in 1 ml 10 m*M* Tris-HCl, pH 10, and disrupted by sonication. The sonicate was boiled for 10 min, centrifuged, and washed once with 1 ml 10 m*M* Tris-HCl, pH 10. The pooled washings were lyophilized, redissolved in 250 μl water, and aliquots (100 μl) analyzed by HPLC. This extraction procedure led to the recovery of >75% of the cellular radioactivity.

CB 3717 polyglutamates were analyzed on a 10×0.46 cm Polygosil 60 5 μm C18 column (Camlab, Cambridge, UK) eluted at a flow rate of 2 ml/minute using a linear gradient from 5 to 16% CH_3CN in 0.1 *M* $NaCH_3COO$, pH 5.0, over 15 minutes and then isocratically at 16% CH_3CN in 0.1 *M* $NaCH_3COO$, pH 5.0, for 5 minutes. Radioactive material present in the column effluent was detected by fraction collection (0.5 ml) with scintillation counting and identified by cochromatography with polyglutamate standards. An example of an analysis is shown in figure 2.

Determination of CB 3717 polyglutamate K_i apparents.—TS activity was determined as previously described (*1*). The enzyme source was the 50,000×*g* supernatant fraction from a mutant L1210 cell line that overproduces TS 45-fold (*6*). The model used for the calculation of apparent inhibitory constant ($K_{i,app}$) was the Goldstein equation applicable to tight-binding inhibitors (Zone B inhibition) (*15*). The Goldstein equation was fitted to the data by a nonlinear least squares regression algorithm (*16*).

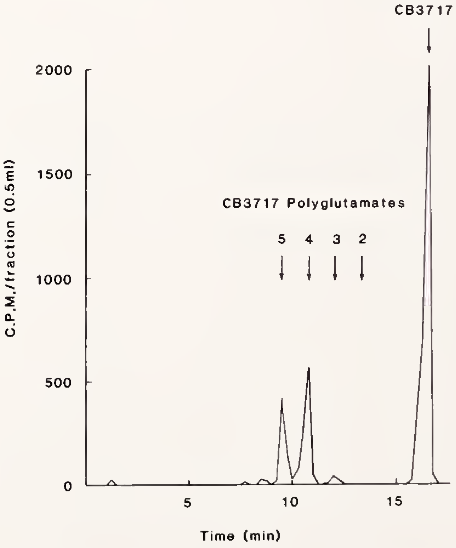


FIGURE 2.—HPLC analysis of an extract of L1210 cells exposed to 50 μM [^{3}H]CB 3717 for 12 hours (——). *Arrows* indicate the position of CB 3717 polyglutamate standards. *See* text for experimental conditions.

Results

As shown in figure 2, exposure of L1210 cells to 50 μM [^{3}H]CB 3717 for 12 hours resulted in the formation of 2 major radioactive components which co-chromatographed with the tetra- and pentaglutamates of CB 3717. The radioactivity associated with these metabolites accounted for approximately 1/3 of the extracted tritium. These results indicate that in whole cells, CB 3717 is susceptible to polyglutamation.

The results for the inhibition of isolated L1210 TS by CB 3717 polyglutamates are given in table 1. One additional glutamate moiety increased the inhibition of TS 26-fold and 2 additional glutamates 87-fold, relative to CB 3717. However, the effect of further polyglutamation was less marked, with 3 and 4 additional glutamate molecules yielding inhibitors 119- and 114-fold more potent than CB 3717, respectively. Thus, CB 3717 polyglutamation serves to markedly potentiate the activity of the drug as a TS inhibitor with 3 additional glutamate residues producing maximal inhibition.

CLINICAL STUDIES

Aims of the Study

The aims of studying CB 3717 given in combination with alkaline diuresis were 3-fold: 1) To develop a regimen which yielded satisfactory urinary alkalinization (approximately pH 8); 2) To determine the effect of the alkalinization regimen on the pharmacokinetics of CB 3717; and 3) To initiate a dose escalation study of CB 3717 given with alkaline diuresis starting at 400 mg/m^2, the recommended phase II dose for CB 3717 given without alkalinization (*2*).

Patients and Methods

CB 3717 disodium salt was supplied by ICI Pharmaceuticals Plc (Macclesfield, Cheshire, UK) and administered once every 3 weeks in $NaHCO_3$, pH 9, as an iv infusion over a period of 30 minutes (400 mg/m^2) or 60 minutes (500 mg/m^2). Patients treated with 400 mg/m^2 received 125 ml 0.15 M NaCl plus 0.5 g $NaHCO_3$/hour for 4 hours prior to the CB 3717 and for 20 hours thereafter. Patients treated with 500 mg/m^2 CB 3717 received 125 ml 0.15 M NaCl plus 0.8 g $NaHCO_3$/hour for 12 hours prior to the CB 3717 and for 12 hours afterwards. The modification of the alkaline diuresis schedule was made to improve the extent of urinary alkalinization achieved (table 2).

Twenty patients have been entered into the trial, 11 at 400 mg/m^2 CB 3717 and 9 at 500 mg/m^2 CB 3717. Patient characteristics are shown in table 3. Patients were monitored for renal function, hepatic toxicity and bone marrow toxicity as reported by Calvert et al. (*2*). Pharmacokinetic studies were performed and the data analyzed as previously described by Alison et al. (*17*), with the exception that individual urine samples were collected and frozen immediately rather than as pooled 24-hour urines.

Results

Urinary Alkalinization

As shown in table 2, in approximately 60% of urine samples collected from patients, using the alkaline diuresis schedule employed with 400 mg/m^2 CB 3717, the pH was 8 or greater. Forty percent of samples were within the pH 7 to 8 range and in only 3 of 52 samples were nonalkaline pH values observed. The alkaline diuresis schedule employed at 500 mg/m^2 gave urines with a pH of >8 in approximately 80% of samples, with only 1 of 48 samples having a pH of <7. Thus, at 500 mg/m^2, the alkaline diuresis schedule developed resulted in satisfactory urinary alkalinization in all patients (mean pH 8.2, range 7.8 to 8.6).

TABLE 1.—Inhibition of L1210 TS by CB 3717 and its polyglutamates

	$K_{i,\ app}$,[a] n$M \pm$ SE	Fold increase in inhibitory activity
CB 3717	26.22 ± 2.23[b]	—
CB 3717 + 1 glu[c]	1.00 ± 0.06	26
CB 3717 + 2 glu	0.30 ± 0.02	87
CB 3717 + 3 glu	0.22 ± 0.01	119
CB 3717 + 4 glu	0.23 ± 0.01	114

[a] Model for calculation of $K_{i,\ app}$ is the Goldstein equation for Zone B inhibition. ±5,10-methylenetetrahydrofolate concentration = 200 μM. Incubated for 1 hour at 37°C.

[b] $K_{i,\ app}$ was determined at 5 concentrations of ±5,10-methylenetetrahydrofolate and was found to decline with decreasing substrate concentration. The inhibition was therefore concluded to be competitive with a K_i of 4 nM (K_i extrapolated to zero substrate concentration) (*7*).

[c] glu = glutamate.

Pharmacokinetics

Plasma CB 3717 levels were studied in 3 patients at 400 mg/m^2 and 4 patients at 500 mg/m^2. An example of 1 patient is given in figure 3. A 2-compartment open model was fitted to the data and the parameters derived are given in table 4.

The urinary excretion of CB 3717 was also studied at both dose levels. As shown in table 2, over the time intervals studied, this route of elimination accounted for approximately 1/4 of the administered dose. At 400 mg/m^2, the major part of the urinary excretion had occurred within 12 hours. Also given in table 2 are the maximum and average urine CB 3717 concentrations recorded.

Toxicities

In 10 patients evaluable for renal toxicity, defined by a decrease in [^{51}Cr]EDTA clearance, there has been only 1 instance of a reduction in glomerular filtration rate to <80% of the pretreatment value. In the other 9 patients, up to 5 courses of CB 3717 have been administered without any marked effect on renal function. Individual glomerular filtration rates are given in figure 4. Furthermore, in 14 evaluable patients following the first course of therapy, there were no elevations in plasma urea levels and only 1 instance in 15 of elevated plasma creatinine (table 5).

Hepatic toxicity, as indicated by raised plasma alanine transaminase and/or alkaline phosphatase levels, remained the toxicity most frequently encountered with CB 3717, with the majority of patients developing some abnormality (table 5). As observed previously in patients given CB 3717 without alkaline diuresis (*2*), hepatotoxicity tended to subside or stabilize after repeated CB 3717 administration and did not limit drug therapy.

Fifteen patients were evaluable for a group of side effects which were collectively defined as malaise. Symptoms used to indicate this side effect included lethargy, aching joints, occasional fever, nausea, and anorexia. Following the first

TABLE 2.—Urinary excretion and urine alkalinization in patients receiving CB 3717 with alkaline diuresis

Dose, mg/m^2	Cumulative urinary CB 3717 excretion, % dose 0–12 hr	Cumulative urinary CB 3717 excretion, % dose 0–24 hr	Peak urine CB 3717 concentration mg/ml	Average urine CB 3717 concentration mg/ml 0–12 hr	Average urine CB 3717 concentration mg/ml 0–24 hr	Mean urinary pH (range)[a]	Distribution of pH values[b] No./Total No. pH 6–7	pH 7–8	pH 8–9
400	17±4[c] (6)	23±5 (5)	0.19 ±0.20 (7)	0.08 ±0.05 (6)	0.06 ±0.02 (5)	8.0 (7.2–8.5)	3/52	18/52	31/52
500	24±4 (3)	—	0.37 ±0.18 (7)	0.17 ±0.07 (3)	—	8.2 (7.8–8.6)	1/48	7/48	40/48

[a] Values are the overall mean urinary pH from 8 patients at each dose level with the range of individual mean pH values.

[b] Frequency of urinary pH values within each of the ranges shown out of the total urine samples collected at each dose level.

[c] Data are mean±SE of (*N*) observations.

course of therapy, 6 of 15 patients experienced no malaise, 3 of 15 had 1 symptom but were not confined to bed for >1 day, and 6 of 15 patients had 2 or more symptoms. In the majority of patients, the malaise was most marked during the second week after therapy. Drug-related vomiting was observed in 4 of 15 patients, being transient in 2 [World Health Organization (WHO) grade 2] and more pronounced in the other two patients (WHO grade 3).

As shown in table 5, only 1 patient suffered from significant hematologic toxicity. He was a man of 62 years with extensive squamous carcinoma of the right maxillary antrum metastatic to a cervical lymph node. Previous therapy included radiotherapy and chemotherapy with vinblastine, MTX, bleomycin, and 2 courses of carboplatin, 400 mg/m^2, the last course having been given 24 days previously. No myelosuppression had been noted during previous therapy. Blood urea and liver function tests were normal at the start of therapy. Seven days after 500 mg/m^2 CB 3717, the WBC had fallen to 0.2×10^9/liter, the platelets to 72×10^9/liter, and the hemoglobin to 64 g/liter. He died the following day, with no postmortem being performed. With the exception of minor reductions in hemoglobin levels, no other hematologic toxicity was observed.

Other miscellaneous toxicities included conjunctivitis in 2 of 15 patients and stomatitis in 1 of 15.

Responses

Although this clinical trial is still at an early stage, objective evidence of antitumor activity has been seen in 1 patient with cervical cancer and 2 patients with ovarian cancer. However, none of these patients reached the criteria necessary to define a partial response.

DISCUSSION

The preclinical studies reported herein provide further insights into the biochemical pharmacology of the TS inhibitor, CB 3717. The demonstration that CB 3717 can undergo polyglutamation in L1210 cells, and the observation that these metabolites are markedly more potent as inhibitors of TS, strongly implicates CB 3717 polyglutamates as the intracellular effectors of CB 3717-induced TS inhibition and, hence, cell growth. In the experiment reported herein, after 12 hours incubation with CB 3717, approximately 30% of the extractable cellular radioactivity could be accounted for as CB 3717 tetra- and pentaglutamate (fig. 2). Given that these metabolites are over 100-fold more potent than CB 3717 as TS inhibitors (table 1), and assuming homogeneous subcellular distribution of CB 3717 and its metabolites, they most probably represent the species responsible for TS inhibition. This observation raises a number of possibilities concerning the mechanism of action of CB 3717 at a cellular level. Although the mode of CB 3717 transport has yet to be determined, intracellular polyglutamation would be predicted to retard the efflux of the drug, by virtue of the increased net negative charge on the molecule. This would in turn prolong the duration of TS inhibition.

TABLE 3.—Characteristics of patients given CB 3717 with alkaline diuresis

Number (M:F)	3:17
Mean age, yr (range)	53 (34–75)
Disease:	
Ovary	12
Cervix	3
Head and neck	2
Miscellaneous	3
Previous therapy:	
None	1
Chemotherapy	10
Chemotherapy + radiotherapy	9
Performance status (WHO):	
1	10
2	6
3	4

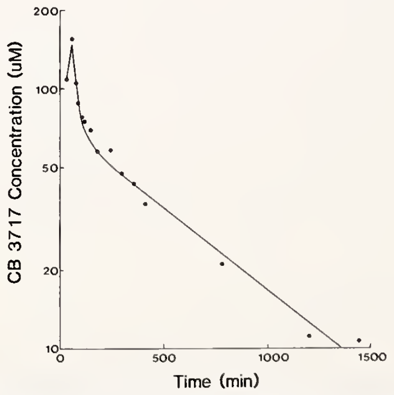


FIGURE 3.—Plasma levels of CB 3717 in a patient treated with 500 mg/m^2 CB 3717. Postinfusion line is a computer-generated fit.

TABLE 4.—Plasma kinetics of CB 3717 administered with alkaline diuresis

Dose mg/m²	t½α, min[a]	t½β, min	V1, liters/m²	Vdss, liters/m²	Clearance, ml/min/m²	AUC, $\mu M \times$hr/m²	Peak plasma concentration, μM
400	21±5[b]	404±39	4.6±1.5	9.7±0.7	19±3	411±50	133±27
500	16±3	422±29	2.9±0.6	9.3±0.8	18±2	563±76	173±10

[a] Pharmacokinetic parameters are as previously defined (*17*).

[b] Data are the mean ± SE of 3 patients at 400 mg/m² and 4 patients at 500 mg/m².

A second consequence of polyglutamation might be to alter, either directly or indirectly, the locus of action of CB 3717 as has been suggested in the case of MTX [(*18–21*) and this volume]. Studies by other workers (*13*) have shown that CB 3717 polyglutamation results in no more than a 2-fold increase in the extent of DHFR inhibition. Hence, CB 3717 is still unlikely to function as a DHFR inhibitor even following polyglutamation. Consistent with this view is the activity of CB 3717 against DHFR overproducing cell lines (*1,5,13*) and the insignificant DHFR elevation in a cell line resistant to CB 3717 (*6*). The possibility remains, however, that CB 3717 polyglutamates could inhibit the folate-dependent enzymes of purine nucleotide biosynthesis. Arguing against this suggestion is the ability of thymidine alone to reverse the cytotoxicity of CB 3717 in a number of cell lines (*1,3,4*) and the inability of hypoxanthine to do so (*13*). Taken together, the available evidence strongly implicates TS as the sole locus of action of CB 3717 with intracellular polyglutamation serving to focus the activity of the drug even more firmly on this enzyme. Studies currently underway are designed to investigate the time course for the formation of CB 3717 polyglutamates and the interrelationship between CB 3717 transport and polyglutamation.

The clinical results of CB 3717 administered with alkaline diuresis indicate that, with the alkaline diuresis schedule employed with 500 mg/m² CB 3717, satisfactory urine alkalinization was achieved in all patients (table 2) and that there was no effect on CB 3717 pharmacokinetics (table 4) (*17*). Thus, there is no reason to believe that any alteration in antitumor activity should occur. In this respect, objective minor responses were seen in the present study. In addition, there was no marked effect on the urinary excretion of CB 3717 (table 2) (*17*). However, the urinary concentrations of CB 3717 observed were below the solubility limits predicted for CB 3717 at the pH values achieved in the majority of patients (i.e., 0.4–4 mg/ml at pH 8 to 9) (table 2). Thus, reduced nephrotoxicity may be anticipated.

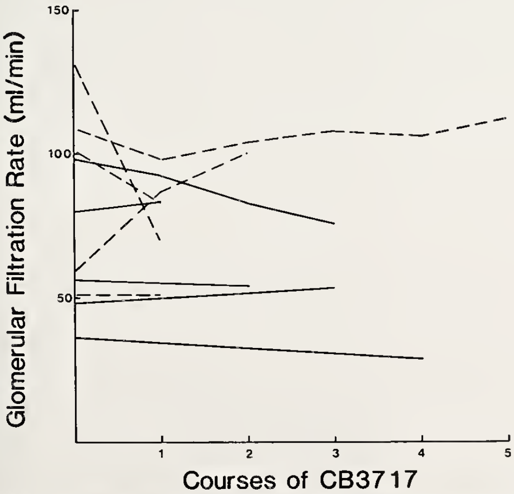


FIGURE 4.—Glomerular filtration rates in patients receiving CB 3717 with alkaline diuresis. Each *line* is an individual patient. Doses of CB 3717: *solid line*=400 mg/m²; *dashed line*=500 mg/m².

With regard to renal side effects, in comparison with CB 3717 given without diuresis (*2*), the incidence of nephrotoxicity may indeed have been reduced by alkaline diuresis. Thus, after receiving CB 3717 at 400 mg/m² without alkaline diuresis, 2 of 9 patients had a drop in glomerular filtration rate to <80% of the pretreatment value. This compares with 0 of 5 in the present study. At 500 mg/m² CB 3717 without alkaline diuresis, 2 of 4 patients had a reduction in glomerular filtration rate to <80% of the pretreatment level, which in 1 patient went below 50%. With alkaline diuresis, 1 of 5 patients suffered impairment of renal function with a reduction to 53% of the pretreatment glomerular filtration rate. Clearly, more patients are required in order to establish the efficacy of alkaline diuresis in preventing CB 3717 nephrotoxicity.

Other toxicities seen when CB 3717 was administered with alkaline diuresis were broadly similar to those seen when the drug was given alone (*2*). That is, hepatotoxicity and malaise were observed with a high frequency and hematologic toxicity, stomatitis, and conjunctivitis were

TABLE 5.—Renal, hepatic and hematologic toxicities of CB 3717 administered with alkaline diuresis (first course)

Toxicity	No. of evaluable patients	No. patients with toxicity grade:[a] 0	1	2	3	4
Hematologic[b]						
Hemoglobin	13	6	4	2	0	1
Leukocytes	15	14	0	0	0	1
Platelets	17	16	0	1	0	0
Renal						
Urea	14	14	0	0	0	0
Creatinine	15	14	1	0	0	0
Hepatic						
Alkaline phosphatase	11	2	5	3	1	0
Alanine transaminase	16	7	5	2	1	1

[a] Toxicity grades as defined by the WHO: Renal and hepatic: 0=≤ 1.25 × N; 1=1.25–2.5 × N; 2=2.5–5 × N; 3=5–10 × N; 4>10 × N, where N is the upper limit of normal.

[b] Hematologic: Hemoglobin (g/100 ml): 0≥11; 1=9.5–10.9; 2=8–9.4; 3=6.5–7.9; 4–<6.5. Leukocytes (1000/mm³): 0≥4; 1=3–3.9; 2=2–2.9; 3=1–1.9; 4=<1. Platelets (1000/mm³): 0>100; 1=75–99; 2=50–74; 3=25–49; 4=<25.

observed occasionally. Although the mechanism of the hepatotoxicity of CB 3717 is unclear, experiments in rodents suggest that it is not due to TS inhibition (*8*). However, the exact relationship, if any, between hepatotoxicity and malaise remains to be established.

In conclusion, the ongoing clinical study of CB 3717 administered with alkaline diuresis indicates that a reduction in nephrotoxicity may be possible. This should allow further dose escalation, which may improve upon the already promising activity of this interesting new antifolate.

REFERENCES

(*1*) Jones TR, Calvert AH, Jackman AL, et al: A potent antitumor quinazoline inhibitor of thymidylate synthetase: Synthesis, biological properties and therapeutic results in mice. Eur J Cancer 17:11–19, 1981.

(*2*) Calvert AH, Alison DL, Harland SJ, et al: A phase I evaluation of the quinazoline antifolate thymidylate synthase inhibitor, N^{10}-propargyl-5,8-dideazafolic acid, CB 3717. J Am Soc Clin Oncol 4:1245–1252, 1986.

(*3*) Jackson RC, Jackman AL, Calvert AH: Biochemical effects of a quinazoline inhibitor of thymidylate synthetase, CB 3717, on human lymphoblastoid cells. Biochem Pharmacol 32:3783–3790, 1983.

(*4*) Jackman AL, Taylor GA, Calvert AH, et al: Modulation of antimetabolite effects: Effects of thymidine on the efficacy of the quinazoline-based thymidylate synthetase inhibitor, CB 3717. Biochem Pharmacol 33:3269–3275, 1984.

(*5*) Diddens H, Niethammer D, Jackson RC: Patterns of cross-resistance to the antifolate drugs trimetrexate, metoprine, homofolate, and CB 3717 in human lymphoma and osteosarcoma cells resistant to methotrexate. Cancer Res 43:5286–5292, 1983.

(*6*) Jackman AL, Alison DL, Calvert AH, et al: Increased thymidylate synthase in L1210 cells possessing acquired resistance to N^{10}-propargyl-5,8-dideazafolic acid (CB 3717): Development, characterization and cross-resistance studies. Cancer Res 46:2810–2815, 1986.

(*7*) Jackman AL, Calvert AH, Hart LI, et al: Inhibition of thymidylate synthetase by the new quinazoline antifolate, CB 3717; enzyme purification and kinetics. *In* Purine Metabolism in Man IV, Part B: Biochemical, Immunological and Cancer Research (De Bruyn C, Simmonds H, Miller M, eds). New York: Plenum Press, 1984, pp 375–378.

(*8*) Newell DR, Alison DL, Jackman AL, et al: Clinical and preclinical pharmacokinetic studies with the thymidylate synthetase (TS) inhibitor N^{10}-propargyl-5,8-dideazafolic acid (CB 3717). Proc Am Assoc Cancer Res 26:350, 1985.

(*9*) Newell DR, Alison DL, Calvert AH, et al: The pharmacokinetics of the thymidylate synthase inhibitor N^{10}-propargyl-5,8-dideazafolic acid (CB 3717) in the mouse. Cancer Treat Rep 70:971–979, 1986.

(*10*) Cantwell B, Macauley V, Harris AL, et al: Phase II study of a novel antifolate, N^{10}-propargyl-5,8-dideazafolic acid (CB 3717) in advanced breast cancer. Proc ASCO 5:63, 1986.

(*11*) Macauley V, Calvert AH, Smith IE: Phase II study of CB 3717 in advanced breast cancer. Br J Cancer 51:597, 1985.

(*12*) Moran RG, Colman PD, Rosowsky A, et al: Structural features of 4-amino antifolates required for substrate activity with mammalian folylpolyglutamate synthetase. Mol Pharmacol 27:156–166, 1985.

(*13*) Cheng Y-C, Dutschman GE, Starnes MC, et al: Activity of the new antifolate N^{10}-propargyl-5,8-dideazafolate and its polyglutamated forms against human dihydrofolate reductase, human thymidylate synthetase, and KB cells containing different levels of dihydrofolate reductase. Cancer Res 45:598–600, 1985.

(*14*) Manteuffel-Cymborowska M, Sikora E, Grzelakowska-Sztabert B: Polyglutamation of the antifolate anticancer drug, N^{10}-propargyl-5,8-dideazafolic acid (CB 3717), in the mouse. Anticancer Res 6:807–812, 1986.

(*15*) Goldstein A: The mechanism of enzyme-inhibitor-substrate reactions. J Gen Physiol 27:529, 1944.

(*16*) Jennrich RI, Sampson PF: Application of stepwise regression to non-linear least squares estimation. Technometrics 10:63–72, 1968.

(*17*) Alison DL, Newell DR, Sessa C, et al: The clinical pharmacokinetics of the novel antifolate N^{10}-propargyl-5,8-dideazafolic acid (CB 3717). Cancer Chemother Pharmacol 14:265–271, 1985.

(*18*) Kisliuk RL, Gaumont Y, Baugh CM, et al: Inhibition of thymidylate synthetase by poly-γ-glutamyl derivatives of folate and methotrexate. *In* Chemistry and Biology of Pteridines (Kisliuk RL, Brown CM, eds). New York: Elsevier/North Holland, 1979, pp 431–435.

(*19*) Allegra CJ, Chabner BA, Drake JC, et al: Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. J Biol Chem 260:9720–9726, 1985.

(*20*) Matherly LH, Barlowe K, Goldman ID: Antifolate effects on folate interconverting and biosynthetic reactions in L1210 cells. Proc Am Assoc Cancer Res 27:254, 1986.

(*21*) Allegra CJ, Hoang K, Baram J, et al: Direct inhibition of de novo purine and pyrimidine synthesis by intracellular (FH_2) following exposure of human cell lines to methotrexate (MTX). Proc Am Assoc Cancer Res 27:258, 1986.

Cisplatin and Dichloromethotrexate: A Pharmacologically Rational Combination[1]

Ronald B. Natale,[2,*] Richard H. Wheeler,[2,3] and James A. Roberts[4]

ABSTRACT—Dichloromethotrexate (DCM), a dihalogenated analog of methotrexate (MTX), is a potent classic folate antagonist that is metabolized and excreted by the liver. It is not nephrotoxic nor are its pharmacokinetics significantly affected by changes in renal function. Therefore, the feasibility of administering a maximum tolerated dose of DCM in combination with high-dose ciplatin (CDDP) was investigated in patients with head and neck, bladder, or cervical cancer—three epithelial carcinomas known to be responsive to single-agent CDDP and MTX. The overall response rates in 24 evaluable patients with head and neck cancer, 21 evaluable patients with bladder cancer, and 22 evaluable patients with cervical cancer were 54%, 57%, and 50%, respectively. Complete response rates of 25%, 19%, and 36%, respectively, were observed. DCM can be administered on a weekly schedule at a maximum tolerated dose in combination with CDDP with tolerable side effects and without significant nephrotoxicity.—NCI Monogr 5:219–223, 1987.

CDDP and MTX are two of the most active single agents in several epithelial carcinomas, including squamous cell carcinoma of the head and neck (*1*), transitional cell carcinoma of the bladder (*2,3*) and squamous cell carcinoma of the cervix (*4*). These drugs possess vastly different mechanisms of action, are largely noncross-resistant and have demonstrated additive-to-synergistic antitumor activity in several animal tumor models (*5,6*). Despite these characteristics, the use of CDDP and MTX in combination poses significant risks of enhanced drug toxicity because both agents are potentially nephrotoxic and lethal if renal excretion of MTX is significantly delayed by CDDP-induced tubular damage (*7–9*). Most clinical trials, therefore, have used doses of CDDP and MTX below their single-agent maximum tolerated doses (MTD), which may be suboptimal, scheduled the drugs at least several days apart, which is inconvenient and could decrease synergistic interactions, or used elaborate hydration-alkalinization schemes, which are burdensome.

However, DCM, a dihalogenated derivative of MTX, is a potent antifol that is metabolized and excreted by the liver, thereby allowing potential concurrent administration with CDDP at full doses. In mice with advanced L1210 leukemia or C3H lymphosarcoma, DCM demonstrated significantly greater antitumor activity than the parent compound. Although it has not undergone broad single-agent clinical trials, DCM appears to have clinical activity similar to MTX. In a comparative trial in patients with Hodgkin's disease or lymphoma, DCM and MTX produced nearly identical response rates (*10*). Major objective responses [complete response (CR) or partial response (PR)] were observed in 1 of 3 patients with hepatocellular carcinoma and in 8 of 12 patients with head and neck cancer treated with DCM intraarterially (*11,12*), in 2 of 21 patients with non-small cell lung cancer treated intramuscularly (*13*), in 2 of 30 patients with acute leukemia treated intravenously (*14*) and, more recently, in 3 of 7 patients with hepatocellular carcinoma treated by weekly 6-hour intravenous infusions (*15*).

We therefore have conducted phase I–II trials of CDDP in combination with a weekly dose-seeking schedule of DCM in patients with advanced head and neck, bladder, or cervical cancers. The results in head and neck cancer and preliminary results in cervical cancer have been previously reported (*16,17*). This paper updates and details results in all 3 tumor types.

PATIENTS AND METHODS

Thirty patients with squamous cell carcinoma of the head and neck, 21 patients with transitional cell carcinoma of the bladder, and 23 patients with squamous cell carcinoma of the cervix were entered into these trials. In all cases, disease was histologically confirmed and objectively measurable. Patient characteristics are detailed in table 1. Additional eligibility criteria included a Karnofsky performance status (KPS)$\geq$50%, WBC counts$\geq$4000 cells/mm^3, platelet count$\geq$125,000 cells/mm^3, creatinine clearance$\geq$50 ml/minute, serum bilirubin$<$1.5 mg/100 ml, no significant cardiac dysfunction, and expected survival of at least 8 weeks. The protocols were approved by our Institutional Review Boards, and all patients gave signed informed consent.

At the start of treatment, all patients had a complete history, physical examination, and laboratory evaluation

---

ABBREVIATIONS: CDDP = cisplatin; CR = complete response; DCM = dichloromethotrexate; KPS = Karnofsky performance status; MTD = maximum tolerated dose; MTX = methotrexate; PD = progressive disease; PR = partial response; SD = stable disease.

---

[1] Supported in part by contract No. N01-CM-07405 (Division of Cancer Treatment) from the National Cancer Institute, and Public Health Service Grants No. 5-MO-1-RR-00042 and T32-CA-09357 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Department of Internal Medicine, Division of Hematology/Oncology, University of Michigan Medical Center, Ann Arbor, MI.

[3] *Present address:* Comprehensive Cancer Center, University of Alabama in Birmingham, Birmingham, AL 35294.

[4] Department of Obstetrics/Gynecology, University of Michigan Medical Center, Ann Arbor, MI.

[*] *Reprint requests to:* Ronald B. Natale, M.D., University of Michigan Hospital, 4701 Upjohn Center, Ann Arbor, MI 48109.

Table 1.—Patient characteristics

	Head and neck	Bladder	Cervical
No. of patients	30	21	23
Age, yr	60 (25–74)	58 (40–79)	62 (35–77)
Males	25	19	0
Females	5	2	23
KPS	60 (40–100)	70 (50–100)	80 (70–100)
Prior therapy			
None	4	5	0
Surgery	22	12	13
Radiation	26	12	21
Chemotherapy	4	0	2
Therapeutic results			
No. of patients unevaluable[a]	6	0	1
No. of patients evaluable	24	21	22
Responses			
CR	6 (25%)	4 (19%)	8 (36%)
PR	7 (29%)	8 (38%)	3 (14%)
MR[b]	0	2	0
SD/PD	11	7	11

[a] Patients with early deaths; 5 patients with inadequate trials.

[b] MR = minor response.

including complete blood and platelet counts, 12-channel biochemical screening profile, urinalysis, 24-hour urine collection for creatinine clearance, chest roentgenogram and EKG. Appropriate radionuclide scans were obtained when the history, physical exam, or laboratory evaluation indicated possible bone, brain, or liver involvement with metastatic tumor. Chest, abdominal and/or pelvic CT scans were done for objective evaluation of the extent of disease and response as indicated. Follow-up studies included physical examinations, complete blood and platelet counts and toxicity notation on each day of chemotherapy administration. A biochemical profile, 24-hour urine collection for creatinine clearance, chest roentgenogram, and repeat radionuclide or CT scan for objective evaluation of tumor response were done with each CDDP administration, which indicated the start of another treatment cycle.

Prior to the start of therapy, patients were classified as good risk or poor risk according to the following criteria. Good risk included patients with KPS≥70%, creatinine clearance>65 ml/minute, hemoglobin≥10 mg/dl, and serum albumin≥3.0 mg/100 ml. Poor risk included patients with KPS<70%, creatinine clearance of 50 to 65 ml/minute, hemoglobin<10 mg/dl, and a serum albumin <3.0 mg/100 ml.

Each treatment cycle began with CDDP given over 30 minutes combined with a 6-hour normal saline and mannitol-induced diuresis and was preceded by vigorous overnight hydration. In patients with head and neck or bladder cancer, the starting dose of CDDP was 100 mg/m^2 for good-risk status and 70 mg/m^2 for poor-risk status. In patients with cervical cancer, the starting dose of CDDP was 90 mg/m^2 for good-risk status and 60 mg/m^2 for poor-risk status. DCM (obtained from the National Cancer Institute) was bolus-injected weekly. The dose of DCM was increased by 100 mg/m^2 weekly until≥grade 1 stomatitis, leukopenia, or thrombocytopenia resulted. Southwest Oncology Group toxicity criteria were used. If the toxicity was grade 1, DCM was continued at that dose; if the toxicity was≥grade 2, the dose was decreased by 100 mg/m^2. In the absence of disease progression or patient refusal, weekly DCM and every-4-week CDDP were given for a total of 4 cycles. Thereafter, CDDP was given every 6 weeks and DCM every 2 weeks.

An adequate trial required a minimum of 1 course of therapy and at least 4 weeks survival. Therapeutic response criteria were defined as follows: CR was the disappearance of all clinical evidence of tumor for at least 4 weeks; PR was a 50% or greater reduction in the sum of the products of the perpendicular diameters of all measurable lesions for at least 4 weeks and no increase in any lesion or appearance of new lesions; stable disease (SD) was a change in measurable lesions that did not qualify for PR or progressive disease, lasting at least 3 treatment cycles (12 weeks) and not accompanied by any new lesions; and progressive disease (PD) was a 50% or greater increase in any measurable lesion. The time to progression, relapse, or death was calculated from the day treatment started.

RESULTS

Therapeutic Responses

Twenty-four of the 30 patients with head and neck cancer, 21 of 21 patients with bladder cancer, and 22 of 23 patients with cervical cancer were considered to be evaluable for response. The unevaluable patients included 2 patients with head and neck cancer who died within 2 weeks of the start of treatment. One patient suffered a carotid hemorrhage and the second died at home of unknown causes but without signs of drug toxicity. Four patients with head and neck cancer failed to complete 1 course of therapy. Three developed recurrent aspiration pneumonias, precluding continued therapy, and one refused further treatment after day 1. One patient with cervical cancer failed to return after the first day of therapy and was lost to follow-up.

Major objective responses occurred in 13 of 24 evaluable patients with head and neck cancer for an overall response rate of 54% (2-sided 95% confidence interval, 33.9% to 75.4%), 12 of 21 evaluable patients with bladder cancer for an overall response rate of 57% (2-sided 95% confidence interval, 33.8% to 78.7%), and 11 of 22 evaluable patients with cervical cancer for an overall response rate of 50% (2-sided 95% confidence interval, 26.4% to 73.6%). Categories of response (CR, PR, etc.) are detailed in table 1.

The median duration of CR (time to relapse) in patients with head and neck cancer was 8.0 months (range, 5.8 to 15 months), with bladder cancer was 22 months (range, 11 to 25+ months) and with cervical cancer was 13 months (range, 7+ to 18+ months). In 2 patients with cervical cancer, complete response was confirmed pathologically on exploratory laparotomy. The median duration of PR in patients with head and neck cancer was 4.2 months (range, 2.1 to 7 months), with bladder cancer was 6.5 months (range, 3.5 to 9.5+ months), and with cervical cancer was 5.8 months (range, 2.5 to 23 months).

Toxicity

Over 300 cycles of combination CDDP+DCM were administered to 74 patients entered into these trials (a "cycle" is defined as 1 dose of CDDP + 4 weekly doses of DCM as occurred during the 4 induction cycles or CDDP + 3 bimonthly doses of DCM as occurred during maintenance). As indicated in table 2, 63 of 74 patients (85%) completed 2 or more cycles of therapy.

Myelosuppression, stomatitis, or both, constituted dose-limiting toxicity in 40%, 32%, and 28%, respectively, of 69 patients in whom DCM was escalated to its MTD. Table 3 details the incidence of myelosuppression and indicates that leukopenia was more common than thrombocytopenia. Notably, despite the weekly escalation of DCM to toxic dose levels, only 6 patients ($<1\%$) developed WBC counts $<1{,}000$ cells/mm^3 and/or platelet counts $<25{,}000$ cells/mm^3. There was 1 death secondary to drug-induced myelosuppression. Table 3 also details the maximum grade of stomatitis observed during the first 4 cycles of therapy. In most cases, stomatitis involved the mouth and nasal passages or resulted in mild diarrhea. There were 2 cases of drug-induced blepharitis and 2 of vaginitis.

Nephrotoxicity was not a significant problem in this study. Serum creatinine increased moderately (1.5- to 2.5-fold) and then returned to pretreatment levels in 10 patients (grade 1 nephrotoxicity), increased and remained elevated (1.5- to 2.5-fold higher than pretreatment levels) in 7 patients (grade 2 nephrotoxicity), and increased and remained more than 2.5-fold higher than pretreatment levels (grade 3 nephrotoxicity) in 1 patient. No patient developed life-threatening nephrotoxicity.

Transient elevation (1.5- to 2-fold increase above pretreatment levels) of serum transaminases (SGOT and SGPT) occurred in two patients, and in 1, the serum bilirubin increased to 1.9 g/dl. In both cases treatment was discontinued because of disease progression, and the abnormal serum chemistry tests returned to normal.

The individual MTD of DCM was defined in 69 of 74 patients entered into these trials and is detailed in table 4. Overall, the median MTD was 600 mg/m^2 (500 mg/m^2 in 26 patients with head and neck cancer, 600 mg/m^2 in 21 patients with bladder cancer, and 700 mg/m^2 in 22 patients with cervical cancer). However, as is true of MTX, patient tolerance of DCM varied greatly and individual MTD ranged from 150 mg/m^2 to 1100 mg/m^2.

The relationship between the MTD of DCM and various factors including patient age, performance status, serum hemoglobin, serum albumin, and creatinine clearance was examined. As has been suggested previously (*18,19*), there was a general correlation with serum albumin (correlation coefficient = 0.41, df = 67, $P<0.001$), although the great variance did not allow serum albumin to be a useful predictor of an individual patient's MTD (fig. 1). Notably, however, 11 of 24 patients with serum albumin <3.8 g/dl experienced dose-limiting toxicity at doses of DCM <500 mg/m^2. This low tolerance of DCM occurred in only 2 of 34 patients with a serum albumin ≥ 3.8 g/dl.

TABLE 2.—Treatment cycles per patient

Tumor type	No. cycles of therapy (CDDP + DCM) ≤1	2	3	4–9	≥10
Head and neck	8	7	7	8	
Bladder	2	4	3	9	3
Cervix	1	3	2	12	5
Total	11	14	12	29	8

TABLE 3.—Maximum toxicity (69 patients)

	No. of patients	Percent of patients
WBC, $\times 10^3/mm^3$		
≥3.0	25	36
2.0–2.9	26	38
1.0–1.9	13	18
<1.0	5	7
Platelets		
≥100,000	44	64
50–100,000	17	25
25–50,000	4	6
<25,000	4	6
Stomatitis		
Grade 0 (normal)	26	38
Grade 1 (erythema)	15	22
Grade 2 (ulcers, able to eat)	21	30
Grade 3 (ulcers, unable to eat)	7	10

CONCLUSIONS

CDDP and DCM are pharmacologically compatible agents. Unlike its parent compound, MTX, DCM can be used at MTD concurrently with CDDP without enhanced drug toxicity and without the need for extraordinary hydration, urinary alkalinization, or leucovorin rescue protocols. In these studies, the incidence of nephrotoxicity did not appear to be greater than would be expected to occur with CDDP alone. Furthermore, CDDP-induced nephrotoxicity did not affect patient tolerance of DCM. CDDP and DCM can be used in combination at nearly maximum single-agent doses with tolerable and manageable side effects.

CDDP plus DCM is an active combination in head and neck, bladder, and cervical cancers. The preliminary results achieved in the latter 2 tumor types are particularly encouraging and are consistent with recent reports of CDDP–MTX combinations in these diseases.

In a Northern California Oncology Group study (*20*), 58 patients with metastatic bladder cancer received 21-day cycles of MTX (40 mg/m^2), vinblastine (5 mg/m^2) on days 1 and 8, and CDDP (100 mg/m^2) on day 2. In the first 22 patients, toxicity was excessive, including 2 deaths from drug-induced renal failure, leukopenia, and sepsis. In the

TABLE 4.—Individual MTD of DCM (69 patients)

Dose, mg/m^2	No. of patients Head and neck	Bladder	Cervix
≤300	3	1	0
400	4	3	2
500	8	4	1
600	4	5	5
700	4	3	8
800	1	2	4
≥900	2	3	2
Total	26	21	22

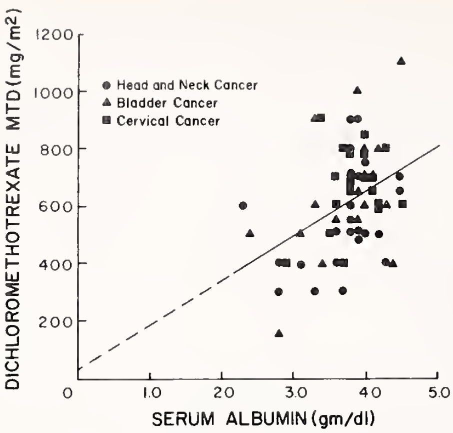


FIGURE 1.—A correlation of albumin concentration (g/dl) and MTD of DCM gives a correlation coefficient of 0.410 with 67 df ($P<0.001$). Slope of the line is 154.

latter 36 patients, MTX and vinblastine were reduced to 30 mg/m^2 and 4 mg/m^2, respectively, and a 24-hour creatinine clearance was required on day 8 (in addition to the 1 required before each dose of CDDP) before MTX was administered. Of 50 evaluable patients, 14 (28%) achieved CR and 14 (28%) achieved PR. The median duration of CR was 9 months. In 3 cases, clinical CR was confirmed on exploratory laparotomy and 1 patient remained alive and disease-free for more than 35 months.

The Memorial group reported results (*21*) in their first 25 patients with metastatic bladder cancer treated with monthly cycles of MTX (30 mg/m^2) on day 1, CDDP (70 mg/m^2), vinblastine (3 mg/m^2) and doxorubicin (30 mg/m^2) on day 2, and MTX and vinblastine repeated on days 15 and 22. Hematologic toxicity was severe (median WBC count nadir of 1600 cells/mm^3), but there was only 1 drug-related death and nephrotoxicity appeared not to be a significant problem. Major objective responses occurred in 17 of 24 evaluable patients (71%) including CR in 12 patients (50%). In 6 cases, CR was confirmed pathologically on surgical exploration, and 8 patients have remained alive for more than 2½ years.

Similarly, the European Organization for Research and Treatment of Cancer treated 53 patients with metastatic bladder cancer with CDDP (70 mg/m^2) on day 1 and MTX (40 mg/m^2) on days 8 and 15 (*22*). Objective responses were observed in 20 patients (46%) including 10 CR (23%) and 10 PR (23%). However, the investigators felt the regimen was "highly toxic" because of excessive myelosuppression, renal dysfunction, stomatitis, and 1 drug-related death.

Vogl et al. treated 9 patients with advanced cervical cancer (*23*) with 21-day cycles of MTX (40 mg/m^2) intramuscularly on days 1 and 15; bleomycin (10 mg) intramuscularly on days 1, 8, and 15; and CDDP (50 mg/m^2) intravenously on day 4. Although an overall response rate of 89% was achieved, severe MTX toxicity developed in several patients due to compromised renal function.

In our studies, overall objective response rates in patients with bladder and cervical cancer were 57% and 50%, respectively. More importantly, 19% of patients with bladder cancer and 36% of patients with cervical cancer achieved clinical complete responses lasting, in some cases, beyond 1½ years. Further testing of CDDP and DCM as a two-drug combination or with the addition of other active agents in randomized comparison against single-agent therapy is indicated in these tumor types.

REFERENCES

(*1*) HONG WK, BROMER R: Chemotherapy in head and neck cancer. N Engl J Med 308:75–79, 1983.

(*2*) YAGODA A: Chemotherapy of metastatic bladder cancer. Cancer 45:1879–1888, 1980.

(*3*) NATALE RB, YAGODA A, WATSON RC, et al: Methotrexate: An active drug in bladder cancer. Cancer 47:1246–1250, 1981.

(*4*) MUSCATO MS, PERRY MC, YARBRO JW: Chemotherapy of cervical carcinoma. Semin Oncol 9:373–387, 1982.

(*5*) PAGE RH, TALLEY RW, BUHAGIAR J: The enhanced antitumor activity of *cis*-diamminedichloroplatinum(II) against murine tumors when combined with other agents. J Clin Hematol Oncol 7:96–101, 1977.

(*6*) BURCHENAL JH, LOKYS L, TURKEVICH J, et al: Rationale of combination chemotherapy. *In* Cisplatin, Current Status and New Developments. (Prestayko AW, Crooke ST, Carter SK, eds). New York: Academic Press, 1980, pp 113–123.

(*7*) OFFERMAN J, MEIJER S, SLEIFER D, et al: Acute effects of *cis*-diamminedichloroplatinum (CDDP) on renal function. Cancer Chemother Pharmacol 12:36–38, 1984.

(*8*) PINEDO HM, CHABNER BA: Role of drug concentration, duration of exposure, and endogenous metabolites in determining methotrexate cytotoxicity. Cancer Treat Rep 61:703–795, 1977.

(*9*) HAIM N, KEDAR A, ROBINSON E: Methotrexate-related deaths in patients previously treated with *cis*-diamminedichloride platinum. Cancer Chemother Pharmacol 13:223–225, 1984.

(*10*) FREI E, SPURR CL, BRINDLEY CO, et al: Clinical studies of dichloromethotrexate (NSC 29630). Clin Pharmacol Ther 6:160–171, 1965.

(*11*) VOGEL CL, ADAMSON RH, DEVITA VT, et al: Preliminary clinical trials of dichloromethotrexate (NSC-29630) in hepatocellular carcinoma. Cancer Chemother Rep 56:249–258, 1972.

(*12*) CLEVELAND JC, JOHNS D, FARNHAM G, et al: Arterial infusion of dichloromethotrexate in cancer of the head and neck: A clinicopharmacologic study. *In* Current Topics in Surgical Research (Quidema GD, Skinner DB, eds), vol 1. New York: Academic Press, 1969, pp 113–120.

(*13*) BAND PR, ROSS CA, HOLLAND JF: Comparison of two dose schedules of dichloromethotrexate (NSC-29630) in lung cancer. Cancer Chemother Rep 57:79–82, 1973.

(*14*) SCHROEDER IR: Clinical trials of 3′,5′-dichloromethotrexate. Proc Am Assoc Cancer Res 3:267, 1961.

(*15*) TESTER WJ, DONEHOWER RC, EDDY JL, et al: Evaluation of weekly escalating doses of dichloromethotrexate in patients with hepatocellular carcinoma and other solid tumors. Cancer Chemother Pharmacol 8:305–310, 1982.

(*16*) WHEELER RH, NATALE RB, ROSHON SG, et al: A phase I–II trial of cisplatin and dichloromethotrexate in squamous cell cancer of the head and neck. J Clin Oncol 2:831–835, 1984.

(*17*) ROBERTS JA, MORLEY GW, NATALE RB, et al: Combination cisplatin and dichloromethotrexate in patients with advanced recurrent cervical cancer: A preliminary report. Gynecol Oncol 19:194–197, 1984.

(*18*) FERNBACH B, TAKAHASHI I, OHNUMA T, et al: Clinical and laboratory re-evaluation of dichloromethotrexate. Recent Results Cancer Res 74:56–64, 1979.

(*19*) OKNOSHI T, OHNUMA T, BROWN JC, et al: Clinical and laboratory studies of dichloromethotrexate given every 6 hours. Proc ASCO 22:353, 1981.

(*20*) HARKER WG, MEYERS FJ, FREIKA FS, et al: Cisplatin, methotrexate and vinblastine (CMV): An effective chemotherapy regimen for metastatic transitional cell carcinoma of the urinary tract: A Northern California Oncology Group Study. J Clin Oncol 3:1463–1470, 1985.

(*21*) STERNBERG CN, YAGODA A, SCHER HI, et al: Preliminary results of M-VAC (methotrexate, vinblastine, doxorubicin and cisplatin) for transitional cell carcinoma of the urothelium. J Urol 133:403–407, 1985.

(*22*) STOTER G, FOSSA SD, KLEIN J, et al: Combination chemotherapy with cisplatin (DDP) and methotrexate (MTX) in advanced bladder cancer: An EORTC phase II study. Proc ASCO 4:106, 1985.

(*23*) VOGL SE, MOUKHTAR M, KAPLAN BH: Chemotherapy for advanced cervical cancer with methotrexate, bleomycin and *cis*-dichlorodiammine-platinum(II). Cancer Treat Rep 63:1005–1006, 1979.

Priority Announcement Service Available for Future Issues

If you would like to be notified when additional *NCI Monographs* are issued, please complete the form below and mail to the address on the form. (You will also be notified when other cancer-related publications are issued.)

MAIL TO: Superintendent of Documents, US Government Printing Office, Washington, DC 20402

Please add my name to your Priority Announcement List (N-569) to be notified when new *NCI Monographs* are issued.

Company or Personal Name

Additional address/attention line

Street address

City State ZIP Code

(or Country)

PLEASE PRINT OR TYPE

MAIL TO: Superintendent of Documents, US Government Printing Office, Washington, DC 20402
Make checks payable to Superintendent of Documents.

Please send me the following volume(s) of *Cancer Treatment Symposia*:

____ copies of the Proceedings of the Workshop on Patterns of Failure After Cancer Treatment (Volume 2, 1983), Stock No. 017-042-00167-6 ($15.00 per copy domestic, $18.75 foreign).

____ copies of The Interdisciplinary Program for Radiation Oncology Research (Volume 1, 1984), Stock No. 017-042-00171-4 ($6.50 per copy domestic, $8.15 foreign).

____ copies of the Proceedings of the Conference on 2′-Deoxycoformycin: Current Status and Future Directions (Volume 2, 1984), Stock No. 017-042-00172-2 ($5.00 per copy domestic, $6.25 foreign).

____ copies of the Proceedings of the Workshop on Mitoxantrone Cardiotoxicity and the Workshop on Clinical Evaluation of Anthracycline Cardiotoxicity (Volume 3, 1984), Stock No. 017-042-00180-3 ($4.75 per copy domestic, $5.95 foreign).

____ copies of the Proceedings of the Third Annual Sanrocco International Meeting on Clinical and Biological Evaluation of the Immunomodifiers (Volume 1, 1985), Stock No. 017-042-00181-1 ($5.50 per copy domestic, $6.90 foreign).

____ copies of the Proceedings of the Workshop on Radiotherapy for Lung Cancer (Volume 2, 1985), Stock No. 017-042-00183-8 ($8.50 per copy domestic, $10.65 foreign).

____ copies of the Proceedings of the National Institutes of Health Consensus Development Conference on Limb-Sparing Treatment of Adult Soft Tissue Sarcomas and Osteosarcomas (Volume 3, 1985), Stock No. 017-042-00185-4 ($6.50 per copy domestic, $8.15 foreign).

____ copies of Compilation of Phase II Results With Single Antineoplastic Agents (Volume 4, 1985), Stock No. 017-042-00189-7 ($22.00 per copy domestic, $27.50 foreign).

Enclosed is $______ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐ - ☐

Order No. __________

MasterCard and VISA accepted.

Credit Card Orders Only
Total charges $______
Fill in the boxes below

Customer's Telephone No.'s
Area Code | Home | Area Code | Office

Credit Card No. ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date Month/Year ☐☐☐☐

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8:00 a.m. to 4:00 p.m. Eastern Time Monday-Friday (except holidays)

Company or Personal Name

Additional address/attention line

Street address

City State ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity		Charges
______	Publications	______
______	Subscriptions	______
	Special Shipping Charges	______
	International Handling	______
	Special Charges	______
	OPNR	______
______	UPNS	
______	Balance Due	
______	Discount	
______	Refund	

NCI Monographs in the JNCI Series

Volumes Available

No. 57: Surveillance, Epidemiology, and End Results Program: Incidence and Mortality Data: 1973–77
#017-042-00156-1; $26.00 (U.S.), $32.50 (elsewhere)

No. 59: Cancer Mortality in the United States: 1950–1977
#017-042-00161-7; $18.00 (U.S.), $22.50 (elsewhere)

No. 61: Third International Symposium: Cancer Therapy by Hyperthermia, Drugs, and Radiation
#017-042-00160-9; $21.00 (U.S.), $26.25 (elsewhere)

No. 64: Management Operations of the National Cancer Institute That Influence the Governance of Science
#017-042-00175-7; $9.00 (U.S.), $11.25 (elsewhere)

No. 65: Use of Small Fish Species in Carcinogenicity Testing
#017-042-00173-1; $16.00 (U.S.), $20.00 (elsewhere)

No. 66: Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens
#017-042-00176-5; $12.00 (U.S.), $15.00 (elsewhere)

No. 67: Selection, Follow-up, and Analysis of Prospective Studies: A Workshop
#017-042-00182-0; $14.00 (U.S.), $17.50 (elsewhere)

No. 68: Multiple Primary Cancers in Connecticut and Denmark
#017-042-00186-2; $23.00 (U.S.), $28.75 (elsewhere)

No. 69: Fourth Symposium on Epidemiology and Cancer Registries in the Pacific Basin
#017-042-00188-9; $16.00 (U.S.), $20.00 (elsewhere)

No. 70: Forty-five Years of Cancer Incidence in Connecticut: 1935–79
#017-042-00187-1; $37.00 (U.S.), $46.25 (elsewhere)

To: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402

Please send me ________ copies of Monograph No. ________, (title) ______________________________

____

Stock # ______________________________; Price ________________

Make checks payable to Superintendent of Documents.

Enclosed is $________ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐-☐

Order No. ______________

MasterCard and
VISA accepted.

Credit Card Orders Only
Total charges $________
Fill in the boxes below

Customer's Telephone No
Area Code — Home — Area Code — Office

Credit Card No. ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date Month/Year ☐☐☐☐

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8:00 a.m. to 4:00 p.m. Eastern Time, Monday–Friday (except holidays).

Company or Personal Name

Additional address/attention line

Street address

City — State — ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity		Charges
______	Publications	______
	Special Shipping Charges	______
	Special Charges	______
	OPNR	______
______	UPNS	
______	Balance Due	
______	Discount	
______	Refund	

GPO

JNCI Monographs

The International Cancer Information Center of the National Cancer Institute announces publication of the first issue of the new *NCI Monographs:* Proceedings of the NIH Consensus Development Conference on Adjuvant Chemotherapy and Endocrine Therapy for Breast Cancer.

During the past decade, systemic therapy has been added to local-regional treatment of breast cancer to prevent recurrence and improve survival of patients. This volume of *NCI Monographs* presents data from many scientists and clinical investigators, who participated in an NIH consensus development conference that was held September 9–11, 1985. Based on these presentations, important conclusions were drawn with respect to improvements in relapse-free survival, appropriate patients for therapy, and the toxic effects of these treatments. Taken together, these data represent one of the most significant advances in the treatment of human solid tumors and should be of interest to all involved in the management of patients with cancer.

Copies of this volume of *NCI Monographs* (Number 1, 1986) are available from the U.S. Government Printing Office ($8.50 per copy domestic, $10.65 foreign). To order, request Stock No. 017-042-00190-1. Make checks payable to the Superintendent of Doucments and mail to: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

To: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402

Please send me ________ copies of Monograph No. ________, (title) ______________________________

____

Stock # ______________________; Price ______________

Make checks payable to Superintendent of Documents.

Enclosed is $________ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐-☐

Order No. ____________

MasterCard and VISA accepted.

Credit Card Orders Only
Total charges $________
Fill in the boxes below

Customer's Telephone No
Area Code | Home | Area Code | Office

Credit Card No. ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date Month/Year ☐☐☐☐

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8:00 a.m. to 4:00 p.m. Eastern Time, Monday–Friday (except holidays)

Company or Personal Name

Additional address/attention line

Street address

City State ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity		Charges
______	Publications	______
	Special Shipping Charges	______
	Special Charges	______
	OPNR	______
______	UPNS	
______	Balance Due	
______	Discount	
______	Refund	

GPO

NCI Monographs

"Cancer Control Objectives for the Nation: 1985–2000" is the second of the new *NCI Monographs* to be published by the International Cancer Information Center of the National Cancer Institute.

This report is the result of the work and collaboration of four Working Groups composed of experts from the United States, Canada, and the staff of the National Cancer Institute. By setting specific objectives, with quantified indicators for measuring progress, and by recommending actions for achieving these objectives, the Working Groups present in this monograph a consensus statement on the potential progress toward cancer control that can be attained by the American public with the help of the concerted efforts of public and private agencies. The goal of a marked reduction in the cancer mortality rate by the year 2000 is a unifying theme of the current cancer control program.

To order copies of this volume (Number 2, 1986) from the United States Government Printing Office, request Stock No. 017-042-00191-9 and enclose payment ($6.00 per copy domestic, $7.50 foreign). Please make checks payable to Superintendent of Documents and mail to: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

To: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402

Please send me ________ copies of Monograph No. ________, (title) ________________________________

Stock # ______________________; Price ______________

Make checks payable to Superintendent of Documents.

Enclosed is $________ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐-☐

Order No. ____________

MasterCard and VISA accepted.

Credit Card Orders Only
Total charges $________
Fill in the boxes below

Customer's Telephone No
Area Code Home Area Code Office

Credit Card No. ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date Month/Year ☐☐☐☐

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8.00 a.m. to 4.00 p.m. Eastern Time, Monday–Friday (except holidays).

Company or Personal Name

Additional address/attention line

Street address

City State ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity Charges
______ Publications ______
Special Shipping Charges ______
Special Charges ______
OPNR ______
______ UPNS
______ Balance Due
______ Discount
______ Refund

GPO

NCI Monographs

NCI Monographs: International Symposium on Labeled and Unlabeled Antibody in Cancer Diagnosis and Therapy

The International Cancer Information Center of the National Cancer Institute announces publication of the third issue of the new *NCI Monographs:* International Symposium on Labeled and Unlabeled Antibody in Cancer Diagnosis and Therapy (Number 3, 1987).

This volume of *NCI Monographs* contains the proceedings of a symposium held at The Johns Hopkins Hospital (Baltimore, MD), which focused on the use of antibodies in cancer diagnosis and treatment. The meeting represents the beginning of dialogue among investigators who have done research in the disciplines of physics, radiobiology, immunology, and pharmacology and those who have clinical expertise in the fields of nuclear medicine and radiation oncology. The work reported at this meeting involves use of dosimetry, low-dose-rate radiation, and antibody fragments, as well as pilot studies of isotope linkage, chemical cytotoxic antibodies, and diagnostic and therapeutic programs. These presentations demonstrate the promise of antibody therapy, which can deliver a broad range of cytotoxic agents, often without the acute side effects of other types of therapy.

Copies of this volume of *NCI Monographs* (Number 3, 1987) are available from the U.S. Government Printing Office ($9.50 per copy domestic, $11.90 foreign). To order, request Stock No. 017-042-00194-3. Make checks payable to the Superintendent of Documents and mail to: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

To: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402

Please send me __________ copies of Monograph No. __________, (title) ______________________________

____

Stock # ______________________________; Price ____________________

Make checks payable to Superintendent of Documents.

Enclosed is $________ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐ - ☐

Order No. ______________

MasterCard and VISA accepted.

Credit Card Orders Only
Total charges $________
Fill in the boxes below

Customer s Telephone No
Area Code | Home | Area Code | Office

Credit Card No ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date Month/Year ☐☐☐☐

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8 00 a m to 4 00 p m Eastern Time Monday–Friday (except holidays)

Company or Personal Name

Additional address attention line

Street address

City   State   ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity   Charges

_____ Publications _____
Special Shipping Charges _____
Special Charges _____
OPNR _____

_____ UPNS
_____ Balance Due
_____ Discount
_____ Refund

GPO

NCI Monographs

Publication of "The First Conference on DNA Topoisomerases in Cancer Chemotherapy" is announced by the International Cancer Information Center.

The proceedings of the first symposium on this topic deal primarily with the interactions of DNA topoisomerases with some of the most effective anticancer drugs such as the anthracyclines, amsacrines, and epipodophyllotoxins. Because resistance to chemotherapy is one of the most challenging problems encountered by clinicians in patient care, the potential benefit of the results of investigations on topoisomerases in cancer cells and the mechanisms of drug resistance provides promise when the transfer of this information will be accelerated into developmental studies of new anticancer drugs. The clinical oncologists, biochemists, and cancer researchers from other scientific disciplines who attended were pleased to hear that so much progress had been made in this new field of cancer research, e.g., in the areas of DNA cleavage mediated by topoisomerase II and the frequency of DNA cleavage in drug-treated cells.

Copies of this volume (Number 4, 1987) may be ordered from the Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402. Please request Stock No. 017-042-00208-7 and enclose payment ($8.00 per copy domestic, $10.00 foreign). Checks and money orders should be made payable to the Superintendent of Documents.

To: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402

Please send me ________ copies of Monograph No. ________, (title) ______________________________

____

Stock # ______________________; Price ______________

Make checks payable to Superintendent of Documents.

Enclosed is $______ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐ - ☐

Order No. ____________

MasterCard and VISA accepted.

Credit Card Orders Only
Total charges $______
Fill in the boxes below

Customer's Telephone No.
Area Code Home Area Code Office

Credit Card No. ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date Month/Year ☐☐☐☐

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8:00 a.m. to 4:00 p.m. Eastern Time: Monday–Friday (except holidays)

Company or Personal Name

Additional address/attention line

Street address

City State ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity		Charges
______	Publications	______
	Special Shipping Charges	______
	Special Charges	______
	OPNR	______
______	UPNS	
______	Balance Due	
______	Discount	
______	Refund	

GPO

NCI Monographs

ONCOLOGY OVERVIEWS

The International Cancer Information Center of the National Cancer Institute announces publication of the following ONCOLOGY OVERVIEWS. ONCOLOGY OVERVIEWS are specialized bibliographies with abstracts, each referencing 150–500 recent publications on a cancer topic of high current interest, drawn from over 3000 sources. Leading researchers in the field covered by each OVERVIEW review and select the most relevant and significant abstracts for each topic, resulting in a tightly focused, quick reference to the most recent cancer literature.

DIAGNOSIS AND THERAPY SERIES

Bone Marrow Transplantation in Cancer,
Stock No. 017-042-00201-0, $5.50

VIROLOGY, IMMUNOLOGY AND BIOLOGY SERIES

Viral Etiology of Cancer II. Papillomaviruses,
Stock No. 017-042-00199-4, $5.50

Viral Etiology of Cancer III. Hepatitis B Virus,
Stock No. 017-042-00197-8, $6.50

Idiotypes, Anti-Idiotypes and Network Regulation,
Stock No. 017-042-00202-8, $6.00

Transforming Growth Factors and Other Autocrine Growth Factors,
Stock No. 017-042-00200-1, $5.00

CARCINOGENESIS SERIES

Translocation and Amplification of Oncogenes,
Stock No. 017-042-00198-6, $6.00

Oncogene Protein Products,
Stock No. 017-042-00205-2, $5.50

Chemopreventive Agents,
Stock No. 017-042-00204-4, $6.50

Potential Occupational Causes of Cancer II. The Urinary Tract,
Stock No. 017-042-00203-6, $4.75

Dioxins and Dibenzofurans in Carcinogenesis, 1980–1986,
Stock No. 017-042-00206-1, $5.50

Radioprotectors: Experimental Studies and Clinical Applications,
Stock No. 017-042-00207-9, $6.50

Superintendent of Documents Publication Order Form

Order Processing Code:
* 6284

Charge your order. It's easy!

1. **Please Type or Print** (Form is aligned for typewriter use.)

All prices include regular domestic postage and handling and are good through 3/88. After this date, please call Order and Information Desk at 202-783-3238 to verify prices.

Qty.	Stock Number	Title	Price Each	Total Price

International customers please add an additional 25% Total for Publications ☐

Please Type or Print

2. ____________________
(Company or personal name)

____
(Additional address/attention line)

____
(Street address)

____
(City, State, ZIP Code)

(   ) ____________________
(Daytime phone including area code)

3. **Please Choose Method of Payment:**

☐ Check payable to the Superintendent of Documents

☐ GPO Deposit Account ☐☐☐☐☐☐☐–☐

☐ VISA, CHOICE or MasterCard Account

☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

____
(Credit card expiration date)

Thank you for your order!

____
(Signature)

4. **Mail To:** Superintendent of Documents, Government Printing Office, Washington, D.C. 20402-9325

5/87

YOU:
Experience and Expertise
PDQ:
State-of-the-Art Cancer Treatment Information
YOU and PDQ:
The Right Combination for Your Patients

A gastroenterologist wants to enter his Dukes C colon cancer patient onto a protocol. By searching PDQ, he finds that a clinical trial is being performed in his city.

A surgeon wants treatment information for a premenopausal woman with Stage II breast cancer; her tumor is estrogen and progesterone receptor negative. PDQ provides standard and investigational treatment options.

A pediatrician wants the prognosis for a child diagnosed as having Wilms' tumor; he thinks it is curable. He learns what the curative regimens are by searching PDQ.

PDQ, the cancer treatment database from the National Cancer Institute, backs up your expertise with

☑ Prognostic, stage, and treatment information for all major types of cancer across the country.
☑ More than 1,000 protocol summaries that are open to patient accrual.
☑ User-friendly menus for easy access to up-to-date information.

To learn more about PDQ, write the National Cancer Institute, International Cancer Information Center, R.A. Bloch Building, Bethesda, Maryland 20892 or telephone (301) 496-7403.

NCI Monographs

A0178
JOURNALS PROCESSING UNIT
NIH LIBRARY
BLDG 10 RM 1L13
1 OF 2

1 Folic Acid Metabolism and Its Disruption by Pharmacologic Agents

9 Unresolved Issues in the Biochemical Pharmacology of Antifolates

17 Biochemical Factors in the Selectivity of Leucovorin Rescue: Selective Inhibition of Leucovorin Reactivation of Dihydrofolate Reductase and Leucovorin Utilization in Purine and Pyrimidine Biosynthesis by Methotrexate and Dihydrofolate Polyglutamates

27 Determinants of Resistance to Antifolates: Biochemical Phenotypes, Their Frequency of Occurrence and Circumvention

37 Folate and Antifolate Cell Binding and Accumulation at Physiologic Folate Concentrations

41 Pharmacokinetics of Leucovorin (D,L-5-Formyltetrahydrofolate) After Intravenous Injection and Constant Intravenous Infusion

47 Absorption Kinetics of Orally Administered Leucovorin Calcium

57 Bioavailability of High-Dose Oral Leucovorin

61 Biochemical and Pharmacologic Rationale for High-Dose Methotrexate

67 High-Dose Methotrexate in Osteosarcoma—Mayo Clinic Studies

71 Methotrexate-Leucovorin Factor Rescue Regimens in Diffuse Large Cell Lymphoma

77 A Randomized Trial of High-Dose Methotrexate Versus Standard-Dose Methotrexate Following Cyclophosphamide, Doxorubicin (Adriamycin), Vincristine, and Prednisone With or Without Bleomycin in the Therapy of Diffuse Large Cell Lymphoma: Preliminary Report of Cancer and Leukemia Group B Study 7851

81 Clinical Pharmacodynamic Studies of High-Dose Methotrexate in Acute Lymphocytic Leukemia

87 Design and Rationale for Novel Antifolates

93 Methodology for the Rational Development of Methotrexate Analogs in the Clinic

99 Therapeutic Synergy of Trimetrexate (CI-898) in Combination With Doxorubicin, Vincristine, Cytoxan, 6-Thioguanine, Cisplatin, or 5-Fluorouracil Against Intraperitoneally Implanted P388 Leukemia

105 Trimetrexate: Clinical Development of a Nonclassical Antifolate

111 Preclinical Biochemical Pharmacology and Toxicology of Piritrexim, a Lipophilic Inhibitor of Dihydrofolate Reductase

121 Initial Clinical Studies of Piritrexim

127 10-Ethyl-10-Deaza-aminopterin: Structural Design and Biochemical, Pharmacologic, and Antitumor Properties

133 Structural Requirements for the Activity of Antifolates as Substrates for Mammalian Folylpolyglutamate Synthetase

139 Folate Analog Nonsubstrates and Inhibitors of Folylpolyglutamate Synthetase as Potential Cancer Chemotherapy Drugs

145 Synthesis and Biologic Activity of New Side-Chain-Altered Methotrexate and Aminopterin Analogs With Dual Inhibitory Action Against Dihydrofolate Reductase and Folylpolyglutamate Synthetase

153 Antifolate Metabolism and Sites of Action: Implications for the Design of New Antifolates

159 Biochemical Rationale for the Synergism of 5-Fluorouracil and Folinic Acid

165 Biochemical and Pharmacologic Basis for Potentiation of 5-Fluorouracil Action by Leucovorin

171 Preliminary Analysis of a Randomized Comparison of 5-Fluorouracil Versus 5-Fluorouracil and High-Dose Continuous-Infusion Folinic Acid in Disseminated Colorectal Cancer

175 A Northern California Oncology Group Randomized Trial of Leucovorin Plus 5-Fluorouracil Versus Sequential Methotrexate, 5-Fluorouracil, and Leucovorin in Patients With Advanced Colorectal Cancer Who Failed Treatment With 5-Fluorouracil or 5-Fluorodeoxyuridine Alone

179 Comparison of Unique Leucovorin and 5-Fluorouracil "Escalating" and "Maximum" Dosage Strategies

185 Clinical Studies of Biochemical Modulation of 5-Fluorouracil by Leucovorin in Patients With Advanced Colorectal Cancer by the North Central Cancer Treatment Group and Mayo Clinic

189 Biologic Modulation of 5-Fluorouracil With High-Dose Leucovorin and Combination Chemotherapy of 5-Fluorouracil and Cisplatin in Metastatic Colorectal Adenocarcinoma

193 Treatment of Advanced Colorectal and Gastric Adenocarcinomas With 5-Fluorouracil and High-Dose Folinic Acid

199 Preliminary Results of a Phase II Trial for the Treatment of Metastatic Breast Cancer With 5-Fluorouracil and Leucovorin

203 A Phase II Trial of 5-Fluorouracil and High-Dose Leucovorin in Gastric Carcinoma and a Phase I Trial of Intraperitoneal 5-Fluorouracil and Leucovorin

207 Transient Acute Hepatotoxicity of High-Dose Methotrexate Therapy During Childhood

213 Recent Preclinical and Clinical Studies With the Thymidylate Synthase Inhibitor N^{10}-Propargyl-5,8-dideazafolic Acid (CB 3717)

219 Cisplatin and Dichloromethotrexate: A Pharmacologically Rational Combination

NIH LIBRARY
3 1496 00329 3894

NIH Publication No. 87-2901 Number 5 1987

NCI Monographs

A Publication
of the National
Cancer Institute

LIBRARY
MAR 1 - 1988
National Institutes of Health

Conference on the Interaction of Radiation Therapy and Chemotherapy

1988
Number 6

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service National Institutes of Health

NCI Monographs

National Cancer Institute, Vincent T. DeVita, Jr., ***Director***
International Cancer Information Center, Susan Molloy Hubbard, ***Director***

Editorial Board

Robert E. Wittes, ***Editor-in-Chief***

Associate Editors

Joseph Aisner
Lewis Aronow
Gregory Curt
Richard L. Cysyk
Ross C. Donehower
Michael Friedman
Timothy J. Kinsella
Robert F. Ozols
Carl Pinsky
David G. Poplack
Richard Simon

Advisory Board

Charles M. Balch
Robert C. Bast, Jr.
Gianni Bonadonna
Edwin C. Cadman
Giulio J. D'Angio
I. David Goldman
Heine H. Hansen
Simon Kramer
Susan E. Krown
John E. Niederhuber
T. L. Phillips
H. M. Pinedo
Carol S. Portlock
Joseph V. Simone
Samuel A. Wells, Jr.

Publications Branch, Robin A. Atkiss, ***Chief***

Monograph Editors

Florence I. Gregoric
Anne McCarthy

Editorial Policy

Manuscripts from key conferences dealing with cancer and closely related research fields, or a related group of papers on specific subjects of importance to cancer research, are considered for publication, with the understanding that they have not been published previously and are submitted exclusively to *NCI Monographs.* All material submitted for consideration will be subject to review, when appropriate, by at least two outside reviewers and one member of the Editorial Board of the *Journal of the National Cancer Institute.* Opinions expressed by the authors are not necessarily those of the publisher or the editors.

Symposia or related papers should be submitted to the Editor-in-Chief, *Journal of the National Cancer Institute,* R. A. Bloch International Cancer Information Center, Building 82, Room 211, National Cancer Institute, Bethesda, MD 20892.

Conference on

The Interaction of Radiation Therapy and Chemotherapy

Williamsburg, Virginia
September 28-October 1, 1986

Major Sponsors:

National Cancer Institute

Bristol-Myers Company

Conference Editors:

Robert E. Wittes

C. Norman Coleman

Program Committee

Chairman:

C. Norman Coleman

Co-chairmen:

- Evan B. Douple
- Todd Wasserman
- Robert E. Wittes

Members:

- Harry Bartelink
- J. Martin Brown
- Bruce A. Chabner
- James D. Cox
- Michael A. Friedman
- John H. Glick
- Anthony E. Howes
- Theodore L. Phillips
- William F. Sindelar
- G. Gordon Steel
- Robert M. Sutherland
- Ian F. Tannock

Faculty:

- Harry Bartelink
- J. Martin Brown
- C. Norman Coleman
- Evan B. Douple
- Michael A. Friedman
- Anthony E. Howes
- Timothy J. Kinsella
- William B. Looney
- Mark E. Nesbit, Jr.
- Robert F. Ozols
- Theodore L. Phillips
- Philip Rubin
- William F. Sindelar
- G. Gordon Steel
- Ian F. Tannock
- Todd Wasserman
- Robert E. Wittes

Conference Summarizer: Theodore L. Phillips

Acknowledgments

The Organizing Committee gratefully acknowledges the support of the following organizations:

Sponsors

Adria Laboratories

Varian, Incorporated

Co-sponsors

American College of Radiology

Inter-Society Council for Radiation Oncology

Benefactors

Burroughs Wellcome Company

Eastman-Kodak Company

Lederle Laboratories

Robert Laboratories

Smith Kline & French Laboratories

Warner-Lambert Pharmaceutical Company

Contributors

Atomic Energy of Canada, Limited
Best Industries Incorporated
Clini-Therm Corporation
E. I. du Pont de Nemours & Company, Incorporated
Eli Lilly & Company
Hoffmann-La Roche Incorporated
Merck Sharp & Dohme Research Laboratories
Siemens Medical Systems, Incorporated

The work was supported in part by Public Health Service grant CA-42012 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

The staff support provided by the American College of Radiology, Philadelphia, PA, is also gratefully acknowledged.

Reviewers

We thank the following reviewers who participated in evaluating the manuscripts submitted to *NCI Monographs* for the proceedings of the Conference on the Interaction of Radiation Therapy and Chemotherapy:

Harry Bartelink
Adrian C. Begg
John E. Biaglow
Dennis M. Brown
J. Martin Brown
Edward Bump
Robert W. Carlson
Bruce A. Chabner
David J. Chaplin
Christopher T. Coughlin
James D. Cox
Evan B. Douple
Ralph E. Durand
Richard G. Evans
Michael A. Friedman
Karen K. Fu
Mitchell H. Gail
Eli Glatstein
John H. Glick
Eric J. Hall
Terence Herman
Anthony E. Howes
Robert F. Kallman
Bruce F. Kimler
Timothy J. Kinsella
John T. Leith
William B. Looney
Douglas F. Martin
Peter Mauch
James B. Mitchell
John E. Moulder
Diana F. Nelson
Mark E. Nesbit, Jr.
Nancy L. Oleinick
Robert F. Ozols
Theodore L. Phillips
Andrew M. Rauth
Philip Rubin
Lawrence V. Rubinstein
Gisele Sarosy
Dietmar W. Siemann
William F. Sindelar
G. Gordon Steel
Fiona A. Stewart
Robert M. Sutherland
Ian F. Tannock
Samuel G. Taylor IV
Margaret A. Tucker
Raul C. Urtasun
Kenneth T. Wheeler, Jr.

TABLE OF CONTENTS

Page

Introduction

C. Norman Coleman 1

Late Toxic Effects in Normal Tissues

Keynote Address: Models of Normal Tissue Injury Following Combined Modality Therapy

Anthony E. Howes 5

Special Lecture: Scoring of Late Toxic Effects—Interaction of Two Modalities

Philip Rubin, Louis S. Constine, and Janice D. Van Ess 9

Interaction of Cisplatin and X-rays in Rat Kidney

Eric van Rongen, Willem C. Kuijpers, and Albert J. van der Kogel 19

Renal Damage in Mice After Treatment With Cisplatin and X-rays: Comparison of Fractionated and Single-dose Studies

F. A. Stewart, A. Luts, Y. Oussoren, A. C. Begg, L. Dewit, and H. Bartelink 23

Effect of Total-body Irradiation With Bone Marrow Transplantation on Toxicity of Cisplatin

John E. Moulder, Brian L. Fish, John S. Holcenberg, and Mary Cheng 29

Effect of Sequencing on Combined Toxicity of Renal Irradiation and Cisplatin

John E. Moulder and Brian L. Fish 35

DNA Damage and Repair in Patients Receiving High-dose Cyclophosphamide and Radiation

Clyde D. Ford and C. Terry Warnick 41

Long-term Consequences of Chemotherapeutic Agents on Hematopoiesis: Development of Altered Radiation Tolerance

C. J. Kovacs, M. J. Evans, J. L. Hooker, and R. M. Johnke 45

Modification of Cyclophosphamide-induced Pulmonary Toxicity in Normal Mice

M. Joan Allalunis-Turner and Dietmar W. Siemann 51

Effect of Continuous Infusion of Bleomycin on Repopulation in Mouse Lip Mucosa During Fractionated Irradiation

L. Vanuytsel, Y. Feng, W. Landuyt, and E. van der Schueren 55

Rodent Model of Chemoradiotherapy-induced White Matter Necrosis

Eugene M. Taylor, J. Russell Geyer, Jerrold M. Milstein, Cheng Mei Shaw, Joseph P. Geraci, Peter Wootton, Bridgette A. Hubbard, and W. Archie Bleyer 59

Quantitative Risk of Second Cancer in Patients in First Complete Remission From Early Stages of Hodgkin's Disease

Michel Henry-Amar 65

Discussion Summary

Anthony E. Howes and Mark E. Nesbit, Jr. 73

Mechanisms of Interaction/Resistance—I

Keynote Address: Mechanisms of Interaction Between Radiation and Drugs With Potential for Improvements in Therapy

Ian F. Tannock and Daniela Rotin 77

Special Lecture: Alternating Chemotherapy and Radiotherapy

William B. Looney 85

Response of Cell Subpopulations in Spheroids to Radiation–Drug Combinations

Ralph E. Durand and Sandra L. Vanderbyl 95

Responses of Tumor Cell Subpopulations to Single Modality and Combined Modality Therapies

Dietmar W. Siemann and Peter C. Keng 101

Increase in Hypoxic Fraction of Human Colon Tumor Xenografts by Preirradiation of Tumor Bed

John T. Leith 107

Development and Application of a Rat Tumor Model for Human Bronchial Carcinoma

H. B. Kal, A. H. van Berkel, B. van der Vecht-de Jong, D. W. van Bekkum, and C. Zurcher 111

Effect of Spirogermanium and Radiation Therapy on the 9L Rat Brain Tumor Model

Bruce F. Kimler, Douglas F. Martin, Richard G. Evans, Robert A. Morantz, and Tribhawan S. Vats 115

Potentiation of Rat Brain Tumor Therapy by Fluosol and Carbogen

Douglas F. Martin, Bruce F. Kimler, Richard G. Evans, Robert A. Morantz, and Tribhawan S. Vats 119

Plasma and Tumor Concentrations of Cisplatin Following Intraperitoneal Infusion or Bolus Injection With or Without Continuous Low-dose-rate Irradiation

Karen K. Fu, Michael W. DeGregorio, and John W. Phillips 123

Platinum Levels in Murine Tumor Following Intraperitoneal Administration of Cisplatin or Paraplatin

Evan B. Douple, Mark D. Totten, and Fred Spencer 129

Improvement of Differential Toxicity Between Tumor and Normal Tissues Using Intratumoral Injection With or Without a Slow-drug-release Matrix System

A. C. Begg, H. Bartelink, F. A. Stewart, D. M. Brown, and E. E. Luck 133

Response of Murine Tumors to Matrix-associated Cisplatin Intratumoral Implants

N. Y. Yu, F. K. Conley, E. E. Luck, and D. M. Brown 137

Effects of Matrix-associated Chemotherapy in Combination With Irradiation In Vivo

A. E. Howes, T. S. Herman, V. P. Montoya, E. E. Luck, and D. M. Brown 141

Administration of Radiation or Cyclophosphamide Versus Alternated Radiation and Cyclophosphamide Treatment of Primary Tumor and Pulmonary Metastases

William B. Looney and Harold A. Hopkins 145

Systematic Investigation Into Interaction of Ionizing Radiation and Doxorubicin in the Dunning R3327G Prostatic Adenocarcinoma Model

Charles F. Gottlieb, G. Burton Seibert, and Norman L. Block 147

Discussion Summary

Ian F. Tannock and William F. Sindelar 155

Mechanisms of Interaction/Resistance—II

Keynote Address: Mechanisms of Cross-resistance Between Radiation and Antineoplastic Drugs

Robert F. Ozols, Hidetaka Masuda, and Thomas C. Hamilton 159

Inhibition of Radiation Dose-rate-sparing Effects in Human Tumor Cells by 3-Aminobenzamide

Lloyd R. Kelland, Lynne Burgess, and G. Gordon Steel 167

Effects of Methylxanthines on Cell-cycle Redistribution and Sensitization to Killing by Low-dose-rate Radiation

Larry E. Dillehay, George Chang, and Jerry R. Williams 173

Interactions Between Antitumor Drugs and Radiation in Mammalian Tumor Cell Lines: Differential Drug Responses and Mechanisms of Resistance Following Fractionated X-Irradiation or Continuous Drug Exposure In Vitro

Bridget T. Hill, Richard D. H. Whelan, Louise K. Hosking, Sharon A. Shellard, Philip Bedford, and Richard B. Lock 177

Cell-cycle, Phase-specific Cell Killing by Carmustine in Sensitive and Resistant Cells

Peter A. Linfoot, Mary Helen Barcellos-Hoff, Thomas P. Brent, Laurence J. Marton, and Dennis F. Deen 183

Chinese Hamster Pleiotropic Multidrug-resistant Cells Are Not Radioresistant

James B. Mitchell, Janet Gamson, Angelo Russo, Norman Friedman, William DeGraff, James Carmichael, and Eli Glatstein 187

Altered Radiosensitivity of Hematopoietic Stem Cells by Vincristine Pretreatment: Superoxide Dismutase Activity as a Possible Mechanism

Roberta M. Johnke, Charles J. Kovacs, Dean P. Loven, Reed S. Abernathy, and Jerry L. Hooker 193

Inhibition of Pentose Cycle of A549 Cells by 6-Aminonicotinamide: Consequences for Aerobic and Hypoxic Radiation Response and for Radiosensitizer Action

Marie E. Varnes 199

Radioresistance of Human Tumor Xenografts: Possible Mechanisms

Shirley Lehnert and Marcelle Guichard 205

Glutathione as a Determinant of Cellular Response to Doxorubicin

F. Y. F. Lee, A. R. Vessey, and D. W. Siemann 211

Cell-cycle Dependence of X-ray Oxygen Effect: Role of Endogenous Glutathione

E. A. Blakely, R. J. Roots, P. Y. Chang, L. Lommel, L. M. Craise, E. H. Goodwin, E. Yee, D. P. Dodgen, and W. F. Blakely 217

Inhibition of Radiation-induced DNA-Protein Cross-link Repair by Glutathione Depletion With L-Buthionine Sulfoximine

Nancy L. Oleinick, Liang-yan Xue, Libby R. Friedman, Laurie L. Donahue, and John E. Biaglow 225

Radioprotection of Rat Subependymal Plate With 4-OH Sodium Butyrate

P. Davey, F. Grieve, and V. A. Ranaldi 231

WR-2721 as Cytotoxic and Radioprotective Agent in Treatment of Murine Lymphoma With Total Body Irradiation

Lawrence R. Coia, Darrell Q. Brown, and Janice Hardiman 235

Discussion Summary

Robert F. Ozols and G. Gordon Steel 241

Combined Modality Treatment—I

Keynote Address: Combined Modality Treatment for Primary Tumor

Harry Bartelink 247

An Organized Multi-institutional Interdisciplinary Evaluation of Role of Radiation Therapy Alone or Combined With Chemotherapy in Treatment of Adenocarcinoma of the Gastrointestinal Tract

H. O. Douglass, Jr., D. M. Stablein, and P. R. M. Thomas 253

Phase III Trial of Irradiation Plus Chemotherapy for Patients With Hepatic Metastases and Hepatoma: Experience of the Northern California Oncology Group

Michael A. Friedman, Theodore L. Phillips, John F. Hannigan, Jr., and Stephen K. Carter 259

Patterns of Failure in Combined Chemotherapy and Radiotherapy for Limited Small Cell Lung Cancer: Southeastern Cancer Study Group Experience

Robert Birch, George A. Omura, F. Anthony Greco, and Carlos A. Perez 265

Use of Hemibody Irradiation as a Non-cross-resistant Agent in Combination With Systematic Chemotherapy in Small Cell Lung Cancer

A. R. Belch, R. C. Urtasun, D. Bodnar, B. Kinney, and R. Amy 271

Ten-year Results of Randomized Trial Comparing Radiotherapy and Concomitant Bleomycin to Radiotherapy Alone in Epidermoid Carcinomas of the Oropharynx: Experience of the European Organization for Research and Treatment of Cancer

F. Eschwege, H. Sancho-Garnier, J. P. Gerard, M. Madelain, A. DeSaulty, A. Jortay, and Y. Cachin 275

Combined Modality Approach to Treatment of Malignant Gliomas—Re-evaluation of RTOG 7401/ECOG 1374 With Long-term Follow-up: A Joint Study of the Radiation Therapy Oncology Group and the Eastern Cooperative Oncology Group

D. F. Nelson, M. Diener-West, J. Horton, C. H. Chang, D. Schoenfeld, and J. S. Nelson 279

Pilot Study of Interaction of Radiation Therapy With Doxorubicin by Continuous Infusion

C. Julian Rosenthal and Marvin Rotman 285

Treatment of High-risk Sarcomas in Children and Young Adults: Analysis of Local Control Using Intensive Combined Modality Therapy

Timothy J. Kinsella, James S. Miser, Timothy J. Triche, Klara Horvath, and Eli Glatstein 291

Integration of Full-dose Adjuvant Chemotherapy With Definitive Radiotherapy for Primary Breast Cancer: Four-year Update

John H. Glick, Barbara L. Fowble, Daniel G. Haller, Ernest F. Rosato, Julius A. Mackie, Clare Weiler, Donna J. Glover, Kevin R. Fox, Shelley Hurwitz, and Robert L. Goodman 297

Comparison of Total Nodal Irradiation Versus Combined Sequence of Mantle Irradiation With Mechlorethamine, Vincristine, Procarbazine, and Prednisone in Clinical Stages I and II Hodgkin's Disease: Experience of the European Organization for Research and Treatment of Cancer

P. Carde, M. Hayat, J. M. Cosset, R. Somers, J. M. V. Burgers, W. Sizoo, J. H. Meerwaldt, A. Hagenbeek, M. Monconduit, E. van der Schueren, J. Thomas, A. Tanguy, E. M. Noordijk, J. Lustman-Marechal, M. Schneider, A. Cattan, J. Michel, B. De Pauw, L. Verdonck, M. Henry-Amar, and M. Tubiana 303

Discussion Summary

Michael A. Friedman and Harry Bartelink 311

Combined Modality Treatment—II

Keynote Address: Platinum-Radiation Interactions

Evan B. Douple 315

Combined Modality Treatment of Peritoneal Mesotheliomas

Gilbert S. Lederman, Abram Recht, Terence Herman, Robert Osteen, Joseph Corson, and Karen H. Antman 321

Continuous Infusion of Etoposide, Bolus Administration of Cisplatin, and Simultaneous Radiation Therapy in Previously Treated Patients With Small Cell Bronchogenic Carcinoma

Kendrith M. Rowland, Jr., Philip Bonomi, Samuel G. Taylor IV, Susan Maffey, Salitha Reddy, and Myung-Sook Lee 323

Concomitant Therapy With Infusion of Cisplatin and 5-Fluorouracil Plus Radiation in Stage III Non-Small Cell Lung Cancer

Samuel G. Taylor IV, Anantha K. Murthy, Philip Bonomi, Salitha Reddy, Myung-Sook Lee, L. Penfield Faber, and Douglas J. Mathisen 327

Phase II Trial of Therapy With Etoposide, 5-Fluorouracil by Continuous Infusion, Cisplatin, and Simultaneous Split-course Radiation in Stage III Non-Small Cell Bronchogenic Carcinoma

Philip Bonomi, Kendrith M. Rowland, Jr., Samuel G. Taylor IV, Mitchell Pincus, Salitha Reddy, Myung-Sook Lee, L. Penfield Faber, William Warren, and C. F. Kittle 331

Combination of Chemotherapy and Radiotherapy in Limited Small Cell Lung Carcinoma: Results of Alternating Schedule in 109 Patients

T. Le Chevalier, R. Arriagada, H. de Thé, H. De Cremoux, M. Martin, P. Baldeyrou, P. Ruffié, F. Benna, M. L. Cerrina, H. Sancho-Garnier, and M. Hayat 335

Simultaneous Therapy With High-dose Cisplatin and Radiation for Unresectable Squamous Cell Cancer of the Head and Neck: A Phase I-II Study

Richard Wheeler, Merle Salter, Suzanne Stephens, Irma Hardy, Glenn Peters, Marshall Urist, and William Maddox 339

Concomitant Therapy With Infusion of Cisplatin and 5-Fluorouracil Plus Radiation in Head and Neck Cancer

Samuel G. Taylor IV, Anantha K. Murthy, John Showel, David D. Caldarelli, James C. Hutchinson, Jr., and Lauren D. Holinger 343

Simultaneous Radiotherapy and Chemotherapy With 5-Fluorouracil and Cisplatin for Locally Confined Squamous Cell Head and Neck Cancer

David J. Adelstein, Vishwa M. Sharan, A. Scott Earle, Ajit C. Shah, Catherine Vlastou, Chandra D. Haria, Susan G. Carter, Catherine Damm, and John D. Hines 347

Sequential Administration of Methotrexate, Cisplatin, and 5-Fluorouracil in Multimodal Therapy for Locally Advanced Head and Neck Cancer

Everett E. Vokes, William J. Moran, Azhar M. Awan, Richard L. Schilsky, Michael Goldman, Harold G. Sutton, Ralph R. Weichselbaum, and William R. Panje 353

Chemotherapy With Low Doses of Radiation Followed by Definitive Radiotherapy for Advanced Unresectable Carcinoma of the Head and Neck

M. Bolla, J. Borgel, A. Guenoun, C. Junien-Lavillauroy, C. Dionet, and C. Vrousos 357

New Strategies for Avoiding Total Laryngectomy in Patients With Head and Neck Cancer

Bhadrasain Vikram, George J. Bosl, David Pfister, William Assad, Elliot W. Strong, Ronald H. Spiro, Roy B. Sessions, Frank P. Gerold, and Jatin P. Shah 361

Clinical Experience With High-dose Cisplatin and Radiation for Treatment of Epithelial Malignancies

Christopher T. Coughlin 365

Tolerance and Preliminary Results of Simultaneous Therapy With Radiation and Cisplatin for Advanced Cervical Cancer

D. J. Monyak, L. B. Twiggs, R. A. Potish, L. L. Adcock, J. E. Savage, G. O. Downey, L. F. Carson, and K. A. Prem 369

Clinical Potential of Synchronous Radiotherapy and Chemotherapy for Advanced Squamous Cell Carcinoma

Madhu J. John, Marshall S. Flam, and Phyllis Ager Mowry 375

Discussion Summary

Timothy J. Kinsella and Evan B. Douple 379

Introduction

C. Norman Coleman[1, *]

The Conference on the Interaction of Radiation and Chemotherapy held in Williamsburg, VA, September 28 to October 1, 1986, was the first international conference dedicated to this subject since the meeting in Hilton Head, SC, 8 years ago. While combined modality therapy is being used in the clinic for virtually every tumor type, the rationale for its use in many situations, other than frustration with the poor therapeutic results of single modality therapy, is not entirely clear. With recent clinical data suggesting that this approach is of value in selected sites and with new advances in basic cancer biology, this meeting was an excellent opportunity to bring together laboratory and clinical researchers.

There are varying rationales for the clinical use of combined modality treatment. Neoadjuvant chemotherapy, also known as "up-front" chemotherapy, is designed to treat micrometastatic disease as soon as possible, before chemotherapy resistance emerges and tumor bulk increases. It also serves as a form of local tumor debulking prior to radiotherapy and/or surgery. No interaction between radiation therapy and chemotherapy is expected. One type of adjuvant therapy employs chemotherapy following local treatment of the primary tumor to eradicate micrometastatic disease. As with neoadjuvant therapy, there is no direct interaction between the modalities, each of which is directed against a different cell population. The modalities can be used simultaneously, with the goal of one enhancing the effects of the other, or radiation therapy and chemotherapy can be used sequentially, with one treatment given after the other is completed. A newer approach is to use partial treatments of each, given in an alternating fashion, so that at the end of the treatment schedule, both will have been given in full dosage. Many classes of drugs are being used in combined modality treatment, and the specifics of drug administration vary in dose, schedule, and route of delivery. Similarly, radiation therapy can be given according to various schedules including standard fractionation, hyperfractionation, accelerated fractionation, and continuous low-dose-rate brachytherapy. Since the permutations and combinations of combined modality therapy are almost limitless, the rationale for a study design and interpretation of results must be based on the best available knowledge.

The reports in this volume were selected from approximately 90 submitted manuscripts after careful peer review. There were five half-day sessions, each with a keynote address and a discussion, which are summarized in these proceedings.

A number of general points about the meeting are worthy of mention.

1) The almost 250 attendees included clinical researchers, basic scientists, and practitioners. While the participation was international, it was clear that the "language barrier" that existed was not among researchers from different countries, but between those doing radiobiology and chemobiology research. Many quite similar concepts are described quite differently. Thus, it seems that a working group should be created to resolve differences in terminology and, to some extent, in experimental design.
2) The therapeutic index, which factors in therapeutic efficacy and treatment toxicity, must be better defined in future clinical trials. While acute effects limit the delivery of the treatment, it is the late effects that may ultimately determine which therapy is tolerable. Although animal models for late effects exist, man is an excellent model to study, particularly if subclinical toxicity can be quantified so that the relative toxicity of different regimens can be compared without harming patients. Therefore, clinical trials must include better assessment of late effects. As noted in these proceedings, efforts to develop a multimodal late effects scoring system for clinical trials are in progress.
3) Combined modality therapy is somewhat unique in that not only have laboratory studies led to clinical trials, but positive clinical trials have given direction to investigation in the laboratory. Despite this excellent level of interaction, it was noted at this meeting and at a preceding meeting sponsored by the National Cancer Institute (Baltimore, MD, September 1985; Theodore L. Phillips, conference chairperson) that combined modality therapy is being used in the clinic in regimens for which there is not necessarily a great deal of supporting data. Since the number of permutations and combinations for the use of this dual modality approach is staggering, only a limited number of clinical trials can be undertaken. Therefore, such trials should not only address an important biologic concept, but must be designed to clearly answer the question posed.
4) Basic knowledge about tumor and normal tissue biology, mechanisms of cell killing, mechanisms of tumor resistance to therapy, and the possible development of cross-resistance between modalities is increasing rapidly. It is expected that this new knowledge will be applied to clinical trials within the next few years.

It was the general feeling of those in attendance that the interaction of radiation therapy and chemotherapy should

---

[1] Joint Center for Radiation Therapy, Harvard Medical School, Boston, MA.

* *Reprint requests:* C. Norman Coleman, M.D., Joint Center for Radiation Therapy, Harvard Medical School, 50 Binney St., Boston, MA 02115.

be the subject of more frequent meetings, so a sequel is being considered for 1989. It is anticipated that the enthusiasm generated by this meeting will lead to increased interdisciplinary activities within the next few years. As chairperson of the Organizing and Program Committee, I would like to acknowledge the excellent work of the committee members, the conference faculty, and the participants. I wish to thank the cosponsors, the Inter-Society Council for Radiation Oncology and the American College of Radiology, for administrative support. The meeting would not have been possible without the financial support of the National Cancer Institute and of the companies listed in these proceedings. Dr. Robert Wittes, his staff, and a long list of reviewers have worked very hard so that these proceedings could be published in a timely fashion. I believe that you will find the papers stimulating. It is hoped that they will serve as a basis for successful laboratory and clinical investigations and as a stimulus for increased interaction between researchers in the basic sciences and in the clinic.

Late Toxic Effects in Normal Tissues

Keynote Address: Models of Normal Tissue Injury Following Combined Modality Therapy

Anthony E. Howes[1,*]

The major objective of combining radiation therapy and chemotherapy into a single course of treatment is to improve tumor response and overall survival of the patient with cancer. Implicit in this objective is that the overall extent of normal tissue injury remains tolerable. However, in the majority of clinical and experimental studies of CMT, relatively little attention has been paid to the actual extent and character of normal tissue damage. This situation is understandable when we consider that 1) increased tumor response is the primary goal of treatment, without which any other effects are essentially irrelevant; and 2) precise description of normal tissue response is often extremely difficult unless only severe, irreversible injury is considered.

The difficulty is illustrated by considering that normal tissue injury following CMT can result in the following types of effects:

1) enhancement of radiation injury by chemotherapy;
2) enhancement of chemotherapy injury by radiation therapy;
3) addition of effects of both modalities on the same organ; or
4) production of injury not usually seen with either modality alone.

Examples of these types of effects are 1) increased incidence of radiation pneumonitis by dactinomycin (*1*); 2) decreased bone marrow tolerance following radiotherapy to the chest wall in the adjuvant treatment of breast cancer (*2*); 3) cardiotoxicity resulting from administration of radiation plus doxorubicin (*3*); and 4) late dementia in patients with small cell undifferentiated carcinoma of the lung who are undergoing chemotherapy and prophylactic whole brain radiotherapy (*4*). Since radiotherapy and many combinations of currently used chemotherapy regimens have the potential to injure practically any tissue type, the complexity of this problem can be readily appreciated.

Initial laboratory approaches to this problem involved the use of relatively large single doses of radiation and drug and evaluation of the incidence of experimental end points of normal tissue injury, usually with lethality as the end point (*5*). The information obtained from such studies is extremely important, not only to alert the clinician to potential risks, but also to allow an estimate of dose modification factors useful in clinical practice. However, the usefulness of such data is severely limited by the simplicity of the experimental models and the complexity of most clinical situations.

At the clinical level, CMT has developed extremely rapidly, often without a proven experimental rationale. Many variations of combination chemotherapy and radiotherapy with or without surgery are currently being used and can be considered to comprise four major treatment strategies.

1) *Adjuvant*—Chemotherapy follows definitive radiation therapy with or without surgery.
2) *Neoadjuvant*—Chemotherapy precedes definitive radiation therapy with or without surgery.
3) *Alternating*—Short sequential courses of chemotherapy and radiotherapy are cycled.
4) *Simultaneous*—Radiation therapy and chemotherapy are given over the same period of time.

All CMT strategies involve multiple drugs, various forms of administration, and multifractionated courses of radiation, often using unconventional techniques. It is, therefore, virtually impossible to consider developing an experimental model that will accurately reproduce any realistic clinical situation. Other, less ambitious, approaches must be pursued. Ideally, the clinical situation in which the responses of each patient's normal tissues are carefully observed and documented should provide the most useful information. This model has, in fact, produced an appreciation of essentially all of the unexpected adverse reactions seen with CMT; for example, 1) leukoencephalopathy following the combination of whole brain irradiation and methotrexate (*6*) and 2) increased risk of leukemia induction in patients with Hodgkin's disease treated with total nodal radiotherapy and chemotherapy with mechlorethamine, vincristine, procarbazine, and prednisone (*7*). Using a clinical model involves 1) choosing normal tissue to be studied and 2) developing a scale of injury that allows accurate and reproducible scoring.

Since, by definition, only reactions occurring above a subclinical threshold can be scored, such systems can rarely give insight into fundamental biologic interactions. With CMT, we are particularly concerned with interactions occurring at the subclinical level that subsequently result in clinically evident injury, so such information is most necessary. A further complication arises from the fact that normal tissue reactions can appear early (during the course of treatment), in which case they can often be successfully managed by modifying the treatment, or late (many weeks after completion of treatment), when they are often pro-

---

ABBREVIATION: CMT = combined modality therapy.

[1] Division of Cancer Biology, Joint Center for Radiation Therapy, Harvard Medical School, Boston, MA.

[*] *Reprint requests:* Anthony E. Howes, M.D., Division of Cancer Biology, Joint Center for Radiation Therapy, Harvard Medical School, 50 Binney St., Boston, MA 02115.

gressive, irreversible, and not amenable to conservative management. It is often the acute reactions that determine how a course of treatment is given, while the late reactions determine its morbidity.

A useful clinical scoring system must therefore be capable of quantitating changes of normal tissue function over time and should also incorporate techniques that allow assay of subclinical changes, so that the overall biologic effect can be assessed. Details of recently proposed late effects scoring systems have been presented in this meeting (*8*). Further information should be provided by new methods of organ imaging involving radioisotope scanning, CT scanning, and nuclear magnetic resonance imaging. Particularly valuable information regarding the combined effects of radiation and doxorubicin on the human heart has been provided by the routine use of endomyocardial biopsies at Stanford University, CA (*9*). With this technique, it has been possible to show not only the extent to which the two modalities interact to produce cardiomyopathy, but also that by modification of the drug schedule, the risk of heart damage can be significantly reduced. Development of similar histological or histochemical techniques for other organs would be of major benefit.

To study the extent and mechanisms of interaction between combined modalities, we must develop and use laboratory models that are simple yet clinically relevant. Despite the ability to culture many different normal tissue cells and even organs, it is doubtful that in vitro systems will be of major benefit for assaying the effects of CMT on normal tissues. There may be some value, however, in the results of studies with density-inhibited, plateau-phase cell systems, which have proliferation kinetic properties similar to those of a late-reacting normal tissue (*10*). Such a system using mouse 10T1/2 cells has been subjected to treatment with fractionated irradiation. Results have been comparable to those obtained with in vivo systems (*11*) and may, therefore, be applicable to studies with CMT. A different problem arises with the development of systems to study the ominous problem of treatment-induced second malignancies. In these studies, the clinical model can only be used to test treatment schemes that are believed, on the bases of theory and/or clinical experience, to be less carcinogenic than prior treatment schemes (*12*). Experimental animal models can provide some information, particularly regarding the carcinogenic potential of individual agents. However, since carcinogenic potentials are generally very low and induced tumors appear relatively late, these systems are generally prohibitively expensive for detailed studies of interaction between modalities. In this situation, the use of in vitro systems to investigate changes in mutation frequency or malignant transformation may prove to be of extreme value. Already, studies of mutation frequency in human cells treated with both x-radiation and an alkylating agent have shown a marked dependency on the order or sequence in which the agents are given (Loeffler J, Little J, personal communication). The results of such studies may indicate how multiple agents should be combined to reduce risk of carcinogenesis to a minimum.

The development of in vivo models of normal tissue injury has been of major concern to radiobiologists for several years. As a result, many small animal systems are now available to study both early and late reactions in a large number of different normal tissues (*13*). One of the most important findings obtained with these models is a clear description of the difference in dose-response relationships between early- and late-responding tissues. Early-reacting tissues, such as those in bone marrow, the gastrointestinal tract, and skin, respond to radiation and many cytotoxic chemicals in a manner that is essentially determined by the number of surviving clonogenic target cells. This is demonstrated by the fact that modification of response with use of functional end points such as de-epithelialization or death from acute toxic effects is directly correlated with assays of stem cell survival, which can be performed with these tissues (*14*). An additional observation is that such early-reacting tissues express damage at a rate that is only slightly influenced by dose, provided that dose is above a threshold to produce a particular lesion. The behavior of such tissues can be schematically represented as comprising three compartments, which have a hierarchical (type H) relationship (*15*): stem cells, which have infinite potential for proliferation but are incapable of function; transit cells, which acquire functional capacity while proliferation becomes increasingly limited; and mature functional cells, which have lost the capacity for proliferation. For type H tissues, therefore, meaningful studies of CMT can be performed by measuring the effect on stem cells, using either a functional or clonal assay. For late-responding tissues and organs such as the central nervous system, the kidney, and the lung, the problem is more difficult. First, there is controversy as to which cells or group of cells is the critical target for determining response (*16*). The relative roles of parenchymal cells versus stromal cells in determining functional response are still unclear. Furthermore, since there are currently no reliable in vivo assays of stem cell survival for these tissues, it is not possible to study relationships between functional and cell-survival end points.

The time after treatment at which functional damage is first expressed in late-reacting tissues, unlike that for early-responding tissues, is highly dependent on dose, with the latent period becoming shorter as the dose of radiation is increased. This observation suggests that there are different mechanisms for the expression of damage by these different tissues. A possible explanation for the behavior of late-reacting tissues is that they consist of functionally competent cells with the potential for proliferation when necessary. Such tissues have been termed flexible, or type F, by Wheldon et al. (*17*), who have shown 1) that after an injury resulting in loss of a large number of cells, the survival cells are depleted, not at a constant rate, as with type H tissues, but at a rate that increases with time; and 2) that at a given level of population size, the instantaneous rate of depletion increases as the dose increases. At any dose level, the time scale of expression of injury is shorter than anticipated from the rate of cell turnover in the unirradiated tissue.

This theoretical model has important implications for studies of CMT. It suggests that there may be no simple relationship between cell survival and a functional end point and that when chemotherapy is combined with radiotherapy, effects on the kinetics of proliferation may play a much more important role than was previously assumed.

Normally, we cannot obtain a dose-response relationship for normal tissue injury for either radiation or drugs in a dose range lower than that required to produce a lesion that can be scored. However, if we treat with several small doses and produce a similar level of injury, we can construct a dose-response curve over the entire range of fractional doses used by assuming that each treatment fraction produces a similar biologic effect (fig. 1). When this technique is applied to data from a wide range of normal tissues (*18*), the curves are found to fit very well to a linear-quadratic equation of the form:

$$\text{effect} = \alpha D + \beta D^2,$$

where D = radiation dose and α and β are constants.

This equation implies that radiation produces at least two types of injury: one that is proportional to dose and consequently does not have capacity for repair and one that is proportional to the square of the dose and has significant but limited repair capacity. The ratio of the constant terms α/β has units of dose and indicates the relative proportions of nonrepairable and repairable damage. Although this model gives no particular insights into the cellular processes leading to eventual loss of functional integrity, it is of immense value in predicting the response of normal tissues to fractionated courses of radiation. One of the most important findings from this model is that the value of α/β for late-reacting tissues is significantly higher than the value for early-reacting tissues. Since it is the late-reacting tissues that often determine the serious morbidity of a course of treatment, we now have a means of studying the effect of chemotherapy on the repair kinetics of normal tissue injury during or following a fractionated course of radiotherapy. By knowing the shape of the dose-response curve and how it is modified by various treatments, we can draw conclusions as to how different agents are interacting (fig. 2). Although we are now in a position to draw some conclusions concerning the effect of chemotherapeutic agents on the kinetics of radiation injury, we are not yet able to build comparable models for normal tissue injury from drugs. As drugs are usually administered systemically, several target tissues are at risk for both early and late reactions. Until more is known concerning the kinetics of repair following chemotherapy, it would not be possible to devise suitable fractionated treatment schemes that might be capable of testing a dose-response model. The difficulty of this problem is illustrated by considering the target tissue for acute lethality in mice following treatment with several different chemotherapeutic agents (*19*). In the majority of cases, deaths occur at approximately 4–8 days after injection. This time scale is similar to that found when the same animals are treated with radiation doses known to produce death from injury to the gastrointestinal tract, and it is often assumed that the drugs are acting on the same target cells. However, whereas this lethality end point correlates well with an in vivo clonal assay for intestinal crypt cell survival with x-rays, similar assays with the drugs show no consistent pattern. This suggests that until more is known about the effects of chemotherapeutic agents on normal tissues at the cellular level, we must focus our research on the ways by which drugs may modify the toxic effects of fractionated irradiation.

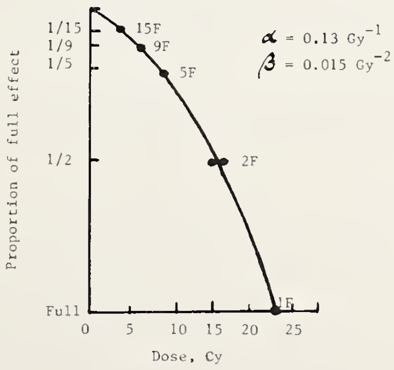


FIGURE 1.—Dose-response curves for mouse skin reconstructed from fractionation experiments, assuming linear quadratic model (*18*).

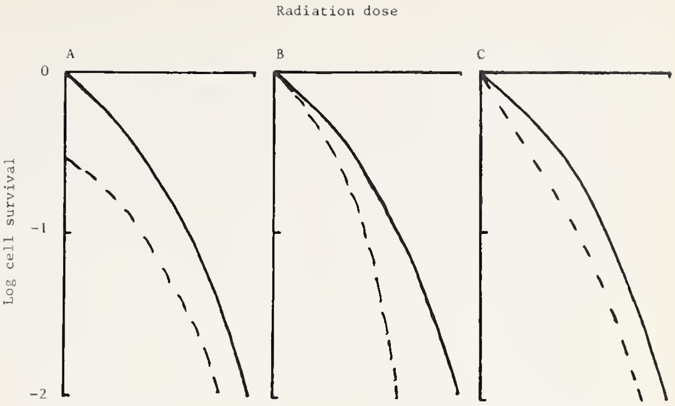


FIGURE 2.—Possible modification of linear quadratic dose-response curve by various forms of interaction between radiotherapy and chemotherapy acting on normal tissue: A) independent cell killing, no change in dose-response curve; B) radiosensitization, predominantly increase in final slope; and C) inhibition of repair of sublethal damage, mainly reduction of shoulder.

In summary, it appears that further clinical trials of CMT will continue at a rapid pace despite a lack of understanding of optimal treatment strategies. We must therefore continue to rely on careful clinical observation, hopefully with the introduction of methods capable of detecting subclinical changes, to provide information concerning the injurious effects of these treatments, in terms of both expected and unexpected toxicity. Our current laboratory models are extremely limited in their ability to predict optimal treatment strategies. However, these models do allow some insight concerning mechanisms of interaction between radiation and drugs, and as further knowledge is gained, it is possible that the current empirical approach to CMT will be replaced by rational and, hopefully, more effective treatment techniques.

REFERENCES

(*1*) PHILLIPS TL: Chemical modification of radiation effects. Cancer 39:987–999, 1977.

(*2*) LEVINE J, COLEMAN CN, COX RS: The effect of postoperative and primary radiation on delivered dose of adjuvant CMF in breast cancer. Cancer 53:237–241, 1984.

(*3*) TORTI FM, BRISTOW MR, HOWES AE, et al: Reduced cardiotoxicity of doxorubicin delivered on a weekly schedule. Assessment by endomyocardial biopsy. Ann Intern Med 99:745–749, 1983.

(*4*) JOHNSON BE, BECKER B, GOFF WB, et al: Neurologic, neuropsychologic, and CT scan abnormalities in survivors of small-cell lung cancer. J Clin Oncol 13:1659–1667, 1985.

(*5*) Phillips TL, Fu KK: The interaction of drug and radiation effects on normal tissues. Int J Radiat Oncol Biol Phys 4:59–64, 1978.

(*6*) Bleyer WA: Neurologic sequelae of methotrexate and ionizing radiation: A new classification. Cancer Treat Rep 65(suppl 1):89–98, 1981.

(*7*) Coleman CN, Williams CJ, Flint A, et al: Hematologic neoplasia in patients treated for Hodgkin's disease. N Engl J Med 297:1249–1252, 1977.

(*8*) Rubin P: Scoring of late toxic effects—Interaction of two modalities. NCI Monogr 6:9–18, 1988.

(*9*) Torti FM, Bristow MR, Howes AE, et al: Reduced cardiotoxicity of doxorubicin delivered on a weekly schedule. Ann Intern Med 99:745–749, 1983.

(*10*) Zeman E, Bedford JS: Changes in early and late effects with dose per fraction. Int J Radiat Oncol Biol Phys 10: 1039–1048, 1984.

(*11*) Zeman E, Bedford JS: Dose rate effects in mammalian cells. Int J Radiat Oncol Biol Phys 10:2089–2098, 1984.

(*12*) Rosenberg S, Kaplan H: The evolution and summary results of the Stanford randomized clinical trials of the management of Hodgkin's disease: 1962–1984. Int J Radiat Oncol Biol Phys 11:5–22, 1985.

(*13*) Hendry J, Fowler JF: Conclusions. Br J Cancer 53: 390–391, 1986.

(*14*) Withers HR, Thames HD, Peters LJ: Differences in the fractionation response of acutely and late responding tissues. *In* Progress in Radio-Oncology (Kärcher KH, Kagelnick HD, Reinert G, eds), vol II. New York: Raven Press, 1982, pp 287–298.

(*15*) Michalowski A, Wheldon TE, Kirk J: Can cell survival parameters be deduced from non-clonogenic assays of radiation damage to normal tissues? Br J Cancer 49(suppl VI):257–261, 1984.

(*16*) Hopewell JW: The importance of vascular damage in the development of late radiation effects in normal tissues. *In* Radiation Biology in Cancer Research (Meyn R, Withers HR, eds). New York: Raven Press, 1980, pp 449–456.

(*17*) Wheldon TE, Michalowski A, Kirk J: The effect of radiation on function in self renewing normal tissues with differing proliferative organisation. Br J Radiol 55:759–764, 1982.

(*18*) Fowler JF: What next in fractionated radiotherapy? Br J Cancer 49(suppl VI):285–300, 1984.

(*19*) Moore JV: The gastro-intestinal syndrome after chemotherapy. Br J Cancer 53(suppl VII):16–19, 1986.

Special Lecture: Scoring of Late Toxic Effects—Interaction of Two Modalities

Philip Rubin,* Louis S. Constine, and Janice D. Van Ess[1]

> "This is the best of times and worst of times."
>
> Charles Dickens

At this moment in time, three decades after the introduction of multimodal treatment for cancer management, one can reflect on the variety of neoplasms that have yielded to this approach. The greatest success has been in the hematologic malignancies where prior to 1950 survival was rare. Now, malignancies such as Hodgkin's disease, some lymphomas (including the diffuse histiocytic variety), and certain leukemias are being cured. In emulation of the success achieved in the treatment of Wilms' tumor, several pediatric tumors have been controlled by the combined use of surgery, radiation, and chemotherapy. Notable in this regard is the treatment of pediatric soft tissue and bone tumors in which more conservative surgery has been combined with the other modalities. By contrast, the radiation-drug combinations have been unsuccessful for most adult solid tumors. As each mode is more aggressively applied, both acute and late toxic effects tend to increase (*1*). Often the patients who were youngest at diagnosis and thus have the longest time to live become the long-term survivors and, ironically, must pay the price of success. In addition to the more well-recognized somatic effects are the loss of fertility (*2,3*), neuroendocrine deficits (*4*), and most distressing, the rise in second malignant tumors (*5*).

Thus, there is considerable promise ("best of times") with regard to combined modality treatment; yet, there is a growing awareness that effective therapy translates into adverse late toxic effects ("worst of times"). In this overview of the scoring of late toxic effects, a series of rhetorical questions will be posed to frame the subject.

QUESTIONS

What are the contrasts and similarities between radiation therapy- and chemotherapy-induced toxicity?

The radiation oncologist has traditionally been concerned with the late toxic effects of treatment (*1*). There is a vast literature that documents the temporal sequence of events as well as the unpredictability of the relationship between acute injury and late effects (*6–8*); that is, the absence of any acute difficulties does not preclude the occurrence of late effects. By contrast, the chemotherapist has been mainly concerned with acute and subacute effects (*9*) because it is this toxicity that determines the tolerance of and thus the ability to administer chemotherapeutic regimens. Thus, a primary difference in the considerations used by chemotherapists and radiation oncologists to determine dose is the belief of chemotherapists that late effects are not a major concern. The focus of the new drug development program of the National Cancer Institute (*10*) has been a series of animal studies conducted in rodents and primates prior to allowing the test compound to be introduced into the clinical arena. When these short-term studies (180 days) are completed and the absence of significant toxic pathophysiological effects is determined, the agent becomes an investigational new drug after an appropriate filing. The occurrence of more chronic adverse effects is, however, not truly assessed. The initiation of medical phase I and phase II studies follows as clinical trials to assess toxicity and efficacy, respectively.

The first awareness that drugs were contributing to late toxic effects occurred in studies of patients undergoing combined modality treatment that included radiation. The dramatic changes that appeared were attributed largely to irradiation and were termed the "recall phenomenon" (*11*). This is compatible with the clinical pathological course paradigm developed by Rubin and Casarett (*12*) and is illustrated in figure 1, in which the concept of residual injury following irradiation is portrayed. According to this model, the degree of recovery or cellular regeneration is never complete, and depending on the level of the initial radiation dose and therefore the level of injury to that vital organ or tissue, the slope of organ cell depletion increases with time. Thus, the subclinical or clinical manifestation progresses and the loss of parenchymal cells increases with time, resulting in accelerated aging or senescence. The addition of chemotherapy (fig. 1A, *vertical dashed lines*) can exacerbate subclinical radiation injury (*curve* B) so that it is clinically expressed (*curve* A). Recognized late radiation effects can shift to a severe or life-threatening, if not fatal, level. Similar clinical or pathological courses are postulated following chemotherapy administration, but the current inability to detect morphological changes in a variety of tissues has falsely given the impression that immediate cellular and ultrastructural changes are temporary and revers-

---

ABBREVIATIONS: TD = tolerance dose(s); CT = computerized tomography; MRI = magnetic resonance imaging; GTDH = grade-time-dose histogram.

[1] Department of Radiation Oncology, Cancer Center, University of Rochester Medical Center, NY.

* *Reprint requests:* Philip Rubin, M.D., Department of Radiation Oncology, Cancer Center, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642.

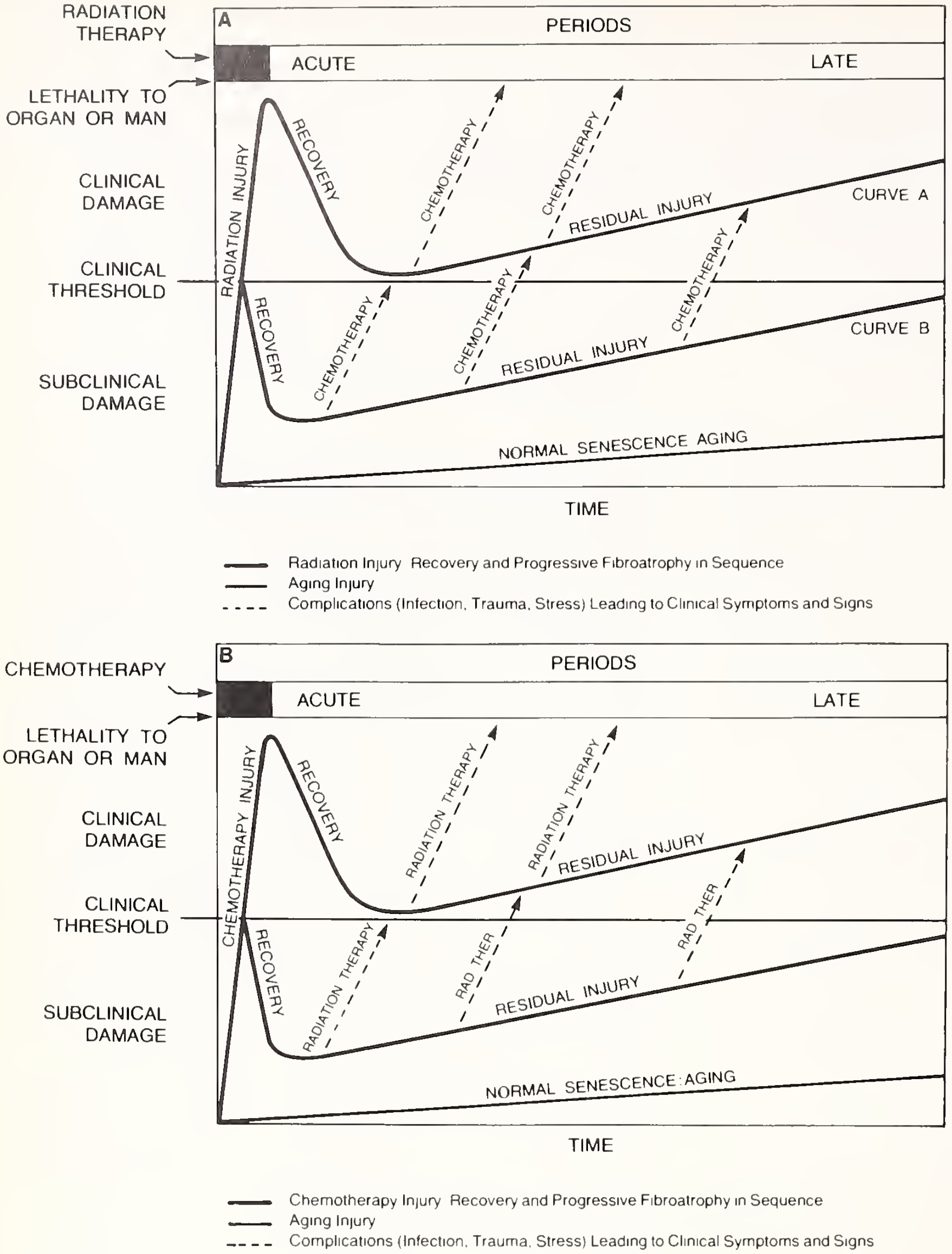


FIGURE 1.—Clinical pathological course of events following radiation exposure can be complicated by addition of chemotherapy, which can augment these changes. Similarly, chemotherapy followed by radiation therapy can result in parallel set of events. Radiation therapy or chemotherapy alone can leave residual injury with sufficient dose. Resulting change is subclinical and may not be observed until addition or subsequent use of seemingly safe dose in other mode. Thus, classically, when radiation therapy precedes chemotherapy (A), introduction of second mode can lead to expression of radiation injury. This is also true of chemotherapy preceding radiation therapy (B). In addition to complications (*dashed lines*) caused by addition of second mode, associated infection, trauma, or stress can lead to expression of overt syndromes or injury. Reprinted with permission from (*12*).

ible (fig. 1B). However, when these chemotherapy-treated tissues are challenged with additional therapy such as irradiation, persistent residual defects are clinically demonstrable (*1,7,13*). These late residual effects have been shown in the laboratory in in vivo experiments in bone marrow, for example (*14*). In addition, late somatic changes due to prolonged drug use have been reported in a number of sites (*9,15–19*).

Further contrasts in regard to the clinical use of radiation therapy and chemotherapy can be demonstrated by considering the concepts of tolerance and therapeutic ratio. In radiation oncology, normal tissue tolerance is defined in terms of a threshold radiation dose that must be exceeded before an effect expresses itself. Dose-response curves for most normal tissue are very steep and occur over a narrow range of 10–50 Gy, which could, depending on the tissue, represent only an increment of 10%–20% of the total dose. In radiation oncology, dose-limiting toxic effects in organs and tissues have been defined with regard to functional significance and characterized in terms of tolerance doses. The minimum tolerance dose (TD 5/5) and the maximum tolerance dose (TD 50/5) refer to severe or life-threatening complications of 5% and 50%, respectively, which occur within 5 years of therapeutic radiation treatment (*12*). To improve tolerance, external irradiation and internal irradiation using intracavitary and interstitial techniques are often combined to take advantage of the low dose rates inherent in internal irradiation. Tolerance to the use of chemotherapy is defined in terms of a maximal tolerated dose and, in this literature, is usually given as a single dose, which is repeated until toxicity is achieved; doses are measured in milligrams per square meter or per kilogram. In the few instances in which late toxic effects are recognized as frequently resulting from prolonged drug usage, a total cumulative dose limit is cited (*9*). Tolerance has been improved by the strategy of using multiple drugs, which have different normal tissue target organs. Modification of drug schedules is widely practiced to heighten the chance that the toxicity will not recur and that the tissue will recover. When 85%–90% of the planned drug schedule is achieved, it is generally considered to be an adequate course, whereas such a dose modification would not be acceptable in radiation therapy. Normal tissue reactions to radiation are defined as severe complication rates, which appear over 5–10 years, whereas acute and subacute toxicity scoring appropriate for normal tissue reactions to chemotherapy is measured in weeks or months.

The concept of rating and scoring toxicity has been a logical derivative of this experience. The most important organs in which to define late effects are the critical dose-limiting vital organs. In radiation oncology, the different categories for late effects in normal tissue stress the degree to which the organ or tissue is vital for survival (*12*). Category I refers to those organs and tissues in which irreparable injury leads to death or very severe morbidity. These include the bone marrow, liver, stomach, intestine, brain, spinal cord, heart, lung, and kidney. Category II includes those organs in which injury is associated with moderate morbidity compatible with survival but resulting in considerable reduction in the quality of life. These organs include the oral cavity, skin, esophagus, rectum, salivary glands, bladder, testes, ovary, cartilage, eye, endocrine glands, peripheral nerves, and ear. In category III are organs in which injury results only from extremely high doses and/or in which loss of function is associated with minimal morbidity; these organs are radioresistant in conventional dose ranges. They include the muscles, lymph nodes and lymphatics, large arteries and veins, uterus, vagina, and breast. These organ categories hold true for chemotherapeutic toxicity in a general sense; however, some exceptions may need to be identified.

What are the end points and diagnostic criteria for scoring toxicity in vital organs?

The end points for scoring toxicity are exemplified by those used for category I vital organs, which are of major concern. The scoring systems used for radiation therapy versus chemotherapy can be contrasted by examining the respective cooperative group criteria. There are approximately 10 cooperative groups in which these criteria have been examined, compared, and synthesized. In radiation scoring, there is a tendency to define a specific pathological effect, compared to the chemotherapy end points, which tend to be functional or physiological. There is more permanence in the abnormal pathological lesions produced by irradiation, compared to the reversible chemotherapy-induced changes, as indicated by the criteria used in grading. The pathological end points and the applicable abnormal physiological or functional end points should be combined. In reviewing the various toxicity scoring systems, one needs to examine the diagnostic criteria, the scale and grades used, and the time and dose dependency of the score, as well as the reversibility and treatability of the injury and the host cofactors (*20*).

In evaluation of the diagnostic injury criteria for toxicity scoring, the end points vary and can be either somatic injury of a vital organ, genetic alterations, or second malignant tumors. The latter are an increasingly important and unfavorable outcome of combined modality regimens. The definition of the end point can be based on a constellation of clinical symptoms and/or signs, laboratory tests, or radiographic imaging, which can include the use of CT and MRI (table 1). The cost and reproducibility of serial studies, particularly those using sophisticated laboratory and imaging procedures, need to be defined. Furthermore, parameters must be established to define whether one, two, or all of the previously mentioned criteria need to be present before the toxic effect is scored. Pathological verification is often essential, particularly for radiation-induced lesions. This would also apply for late effects due to chemotherapy, with or without irradiation. It must always be recognized that recurrent tumor can often mimic, be associated with, and even produce late effects.

There is general agreement that the scale of toxicity scoring should include five grades: mild, moderate, severe, life-threatening, and fatal (table 2). It can be determined that function is decreased by a certain percentage if an appropriate enzyme or another biochemical marker related to the normal tissue function can be defined and rated for change. It is clear that some tissues will not have grade 4 or 5 features, and it is for this reason that they would be relegated to the categories II and III for normal tissues. For end-results reporting, the scores should be concerned with severe or life-threatening toxic effects; namely, grades 3–5. Reversibility and treatability are important factors influencing grade assignment. Reversible physiological lesions are scored lower than are permanent pathological injuries. The grade is related to whether the lesions are correctable only by surgical methods, which would lead to a higher score than the score for those that are medically correctable (table 1). Implied in these corrective therapies is the fact that the lesions are generally reversible or transitory and are thus medically manageable, or they are permanent, requiring surgical resection. There are, of course, exceptions to this operational definition; for example, when medical or hormonal therapy is necessary on an ongoing basis, then the responsible injury is almost certainly a permanent one.

What is the impact of other therapeutic modalities on toxicity grade?

The contribution to toxicity by other modalities in addition to radiation therapy and chemotherapy must also be considered, although these two modes have naturally been the focus of major concerns in cooperative group activities. Surgery has long been known to complicate the use of irradiation and vice versa, and this must be factored into any consideration or tabulation of effects. Surgery undertaken prior to the use of irradiation will reduce radiation tolerance because of the resulting injury to normal tissues and their vascular supply. Surgery undertaken after high-dose radiation therapy may be even more hazardous, since tissue healing is impaired, leading to more frequent wound complications. The radiation dose and the time interval between surgery and irradiation are crucial. Doses $\leq$4,500–5,000 cGy are well tolerated, and a planned interval of 4–6 weeks between irradiation and subsequent surgery allows for excellent healing.

TABLE 1.—Class I/II organs: pathological lesions, evaluative testing, and possible interventions for treatment-related injuries[a]

Organ	Class	Lesion	Evaluative testing	Therapeutic intervention		Scoring/ grading
				Medical	Surgical	
Nervous system						
Peripheral	II	Demyelinization, hepatic necrosis	Nerve conduction studies	Anesthesia (e.g., nerve block)	Surgery	A = L
Central	I					
Brain		Demyelinization, necrosis	CT/MRI, EEG MBP in CSF/serum[b]	Steroids Antiepileptics Supportive care	Surgery	A ≠ L
Spinal cord		Demyelinization, necrosis	Myelogram, MRI MBP[b]	Steroids Antiepileptics Supportive care		A ≠ L
Bone marrow	I	Aplasia, fatty replacement	Peripheral blood counts Bone marrow scan, MRI[b] Bone marrow biopsy/ aspiration	Transfusion Antibiotics	Bone marrow transplant	A = L
Lung	I	XRT Acute toxic effects: pneumonitis, alveolar cell hyperplasia, intra-alveolar hyaline membrane, thickened alveolar septum Chronic toxic effect: fibrosis Chemo Carmustine: interstitial pneumonitis, pulmonary fibrosis Cyclophosphamide: proliferation of alveolar lining cells Busulfan: interstitial fibrosis	Pulmonary function test (including exercise testing), CXR, CT, isotope scans, biochemical tests in serum or lung lavage (surfactant)[b]	Steroids O_2-assisted ventilation Surfactant[b]		A = L
Heart	I	XRT Pericarditis, endomyocarditis, pancarditis, vascular changes Chemo Anthracycline: myopathy	ECG, CXR, ECHO, rest/exercise gated nuclear angiography, endomyocardial biopsy, thallium scan	Medications	Surgery (e.g., for tamponade)	A ≠ L
Liver	I	Hepatitis, veno-occlusive disease, cirrhosis	SGOT, SGPT, alkaline phosphatase, bilirubin, nuclear scans, CT/MRI, ultrasound	Medications	Resection	A = L
Kidney	I	Acute nephritis, chronic nephropathy, atrophy	Serum: BUN, creatinine, albumin Urine: protein, blood Blood pressure, CT, nuclear scans, prostaglandin excretion[b]	Medications Dialysis	Transplant	A = L
Skin	II	Inflammation, ulceration, epilation, atrophy		Topical medications Wet/dry soaks Supportive care	Surgery	A ≠ L
Subcutaneous tissue	II	Fibrosis, atrophy		Supportive care Exercise (e.g., physical therapy)		A ≠ L

TABLE 1.—Class I/II organs: pathological lesions, evaluative testing, and possible interventions for treatment-related injuries (*continued*)[a]

Organ	Class	Lesion	Evaluative testing	Therapeutic intervention		Scoring/ grading
				Medical	Surgical	
Oral cavity	II					
Mucosa		Inflammation, ulceration, fibrosis		Medications Supportive care		A = L
Bone		Osteonecrosis	X-rays	Supportive care	Surgery	L
Salivary glands		Inflammation, fibrosis, atrophy	Sialogram	Supportive care Artificial saliva		A = L
Gastrointestinal	I					
Esophagus		Inflammation, fibrosis, necrosis	Radiography: plain films with contrast agents, barium swallow, fluoroscopy, CT/MRI Esophagoscopy	Medications Supportive care	Surgery	A ≠ L
Stomach		Inflammation, ulceration, atrophy	Radiography, endoscopy, barium swallow	Medications Supportive care	Surgery	A = L
UGI		Inflammation, ulceration, fibrosis, necrosis	Radiography: plain films with contrast agents Endoscopy, stool guaiac	Medications Diet	Surgery	A = L
LGI		Inflammation, ulceration, fibrosis, necrosis	Radiography: plain films with contrast agents Endoscopy, stool guaiac	Medications	Surgery	A = L
Bladder	II	Telangiectasia, fibrosis, necrosis	Radiography with contrast agents Cystoscopy, IVP, RUG	Medications	Surgery	A = L
Ears	II					
Hearing		Inflammation, serous otitis, sclerosis	Auditory testing	Medication Mechanical device	Surgery	A = L
Balance		Labyrinthitis	Equilibrium testing	Medication	Surgery	A = L
Eye	II					
Retina		Retinitis, vascular changes, detachment, fibrosis	Ophthalmic examination, evoked responses	Medication	Surgery	A = L
Conjunctiva		Conjunctivitis, telangiectasia, ulceration	Ophthalmic examination	Medication		A ≠ L
Cornea		Keratitis, neovascularization	Ophthalmic examination	Medication		A ≠ L
Angle of Schlemm		Glaucoma, aqueous veins	Ophthalmic examination, tonometry	Medication	Surgery	A = L
Lens		Cataract	Ophthalmic examination		Surgery	L
Cartilage	II					
Growing		Growth arrest	Sequential measurements, x-rays	Mechanical lifts	Epiphysectomy of contralateral epiphysis	L
Mature		Chondritis, loss of vascularization, cellular disarray, necrosis	X-rays including angiography		Surgery	A = L
Bone	II	Osteonecrosis	X-rays	Supportive care	Surgery	L
Endocrine glands	II					
Hypothalamus/ pituitary		Cellular changes: degranulation, vacuolation, necrosis, hemorrhage	Baseline hormonal and stimulation testing	Medication		A = L
Thyroid		Atrophy	Baseline hormonal and stimulation testing	Medication		A = L

[a] Diagnostic procedure is always biopsy, and physical examination is always performed. A = acute toxic effects; L = late toxic effects; MBP = myelin basic protein; XRT = radiation therapy; CXR = chest x-ray; Chemo = chemotherapy; ECHO = echocardiogram; UGI = upper gastrointestinal series; LGI = lower gastrointestinal series; IVP = iv pyelogram; RUG = retrograde urogram. [b] New testing procedures are being developed.

TABLE 2.—Toxicity scoring[a]

	Grade				
	1 Mild	2 Moderate	3 Severe	4 Life-threatening	5 Fatal
Increase or decrease	10%	25%	50%	75%	100%
Range of values	1–3	3–5	5–8	8–10	>10
Multiple of normal value	1.5	3	5	10	>10

[a] Figures assign numerical values in intuitive way to define changes in body functions measured to determine degree of toxicity. These are defined in many ways depending on test in question. In this example, they are defined as percent increase or decrease in value or as multiple of normal values.

Biologic response modifiers, immunosuppression, and immunocompatibility need to be mentioned. For example, the development of interstitial pneumonitis following irradiation, which is often fatal in the setting of total body irradiation, is less frequent and severe if syngeneic rather than allogeneic bone marrow is used in the transplantation process (*21*). This may relate to the contributions of graft-versus-host disease or other immunologic or infectious factors in the allogeneic situation. Other host cofactors, in addition to recurrent tumor, alter the baseline functioning of normal organs and tissues and may lower tolerance to intensive treatment by radiation or drugs. Associated diseases of senescence such as chronic obstructive pulmonary disease and atherosclerotic cardiac impairments would compromise the ability for the associated tissues to withstand full-dose treatment as readily. Diabetes, infection, and trauma are all known to heighten radiation reactions and in certain circumstances would act in an equally unfavorable manner for organs that are vulnerable to chemotherapy (*22,23*). A notable example is chronic obstructive pulmonary disease in smokers, which lowers tolerance to pulmonary irradiation and to such drugs as bleomycin.

Is there an optimal dose-time relationship for radiation therapy and chemotherapy?

Dose intensity and likewise the time over which that dose is given need to be carefully considered. In radiation oncology, dose-time factors are critical and are determined by the size of the individual doses and the time interval between fractions. Dose fractionation schedules are typically five daily treatments with 2 days of rest on weekends, but there has been an increased tendency to give multiple fractions daily: hyperfractionation and/or accelerated fractionation. The rate of irradiation must be considered from the viewpoint of normal tissue tolerance. Protraction or lowering the dose rate to 0.1–0.01 Gy/minute improves tolerance and allows for delivering daily doses of 1,000 cGy. In contrast, high dose rates of 10–100 cGy/minute, commonly used in external beam therapy, permit a 200-cGy daily dose or 1,000 cGy/week. Split-course schedules are generally considered inferior to continuous conventional fractionation regimens.

Again, in contrast to radiation therapy regimens, in which dose-time alterations are rather limited and infrequently utilized, dose-time modifications in chemotherapy programs are very common and highly dependent on the toxicity scoring and grades. There is a general tendency in most protocols to modify the dose of chemotherapy, depending on acute toxicity such as hematologic suppression. Recent investigations into therapeutic efficacy and toxicity support maintenance of the optimal fractional dose size in chemotherapy and, when necessary, lengthening of the time interval between cycles. Such a recommendation can be found in the analysis of level I–III chemotherapy in postmenopausal breast cancer patients by Bonadonna and Valagussa (*24*). Other possible variations include delivery by bolus versus continuous infusion, which alters the drug delivery concentrations, changing toxicity as well as tumor responsiveness. Weekly and monthly cycling is also very common in chemotherapy schedules, which are often administered in split-course regimens, in contrast to radiation therapy. In combination chemotherapy-radiotherapy programs, the optimal use of one modality can compromise the ideal use of the other. Nevertheless, such disadvantages may be generally outweighed by the benefits.

Is there a safe dose of radiation or chemotherapy?

Dose-limiting organs and tissues in radiation oncology have been defined in terms of their tolerance doses (TD 5/5 and TD 50/5). Doses below the TD 5/5 are considered to be "safe doses" but are affected by the relative volume of the organ irradiated (*12*); that is, irradiation to a portion of an organ is generally better tolerated than irradiation to the entire organ. Thus, the concept of dose-volume histograms is helpful in weighing "volume" in the therapy decision. The values that have been generally recognized as radiation TD are conditioned by volume. This, however, assumes that *no other* competing modalities such as chemotherapy or surgery will be employed. There are only a few chemotherapeutic agents for which cumulative doses or total doses within a certain time period have been determined as upper limits for tolerance; such determinations are common in administration of radiotherapy. Doxorubicin and carmustine are good examples of drugs for which maximal accumulated dose levels are defined in terms of tolerance (*25,26*).

There is a trend toward the wide use of combined modalities, despite the fact that there is no truly safe dose of either radiation or chemotherapy. Radiation administration is known to affect the interstitium and the fine vasculature of the heart, leading to increased connective tissue thickening with minimal direct effects on cardiac myocytes (*27*). Doxorubicin is known to vacuolate the cardiac myocyte, with little change in the interstitium (*28*). A seemingly "safe low dose" in either of these modalities may be well tolerated; for example, in patients with mediastinal Hodgkin's disease. However, when both modalities are applied, an additive effect occurs, leading to cardiac decompensation (*28,29*) and cardiomyopathy. This has been documented both in pediatric (*30*) and adult (*29*) oncology and can even occur when many years separate the administration of presumed safe doses of either agent. Although recovery, repair, and regeneration are known to occur after treatment, a residual injury persists subclinically and is unmasked by the administration of the other mode. It is clear that the concept of a safe dose or a "dose within tolerance" no longer applies in this multimodality era.

Is there a difference between an acute and a late toxic effect?

Time is perhaps more critical than dose in the consideration of end point criteria for late toxic effects. Acute toxicity is defined as effects that occur during or shortly after the administration of radiation therapy or chemotherapy. The usual curative radiation therapy course is administered over 4–8 weeks, whereas palliative treatment is given over a shorter time period. Chemotherapy schedules, however, continue to cycle for longer periods of time, lasting 6 months to 1 year. Simply stated, acute toxic effects can be defined as those that develop within 6 months of treatment, and late toxic effects are those that can become evident from >6 months to many years after treatment is finished. Although this time frame is applicable to radiation therapy, it becomes somewhat muddied when applied to the more protracted chemotherapy regimens. Furthermore, the time interval to development of second malignant tumors is a minimum of 5 and perhaps ≥10 years (*5*).

A number of paradoxical situations implicit in the previous discussion arise since the definitions of acute versus late toxicity, as adopted from radiation therapy experiences, become confused with the subsequent application of chemotherapy in the same patient. Expanding on the analogy of the combined use of doxorubicin and irradiation for a patient with mediastinal Hodgkin's disease, the dilemma is illustrated in figure 2. Let us construct a case of mediastinal Hodgkin's disease in which irradiation was administered to a volume including the heart, and a decade later, the disease recurred. If doxorubicin were part of the multidrug combination utilized for salvage therapy, a subclinical cardiac effect caused by radiation could be translated into clinical toxicity, which might even become fatal if large enough doses were used (fig. 2, *dashed oblique lines*). Several questions remain unanswered. Is the cardiac decompensation or fatality considered *an acute effect of chemotherapy* or a *late effect of radiation therapy?* Should the toxic effect be scored from the time of radiation administration or from the time the last chemotherapy dose is given in the planned regimen? If a chemotherapy program continues for 1 year with crossover to another non-cross-resistant drug combination, are the chemotherapeutic toxic effects, which become cumulative, considered to be acute in the sense that the treatment is still ongoing, or are they late in the sense that >1 year may have elapsed between initial therapy and the time that the accumulated effect clinically expresses itself in a more permanent fashion (fig. 3)?

Is grade alone enough to accurately score late toxic effects?

The grade of toxicity varies as a function of time in the clinical pathological post-therapy course (fig. 4). With many acute effects, the toxicity reaches a peak, and it is the peak score that is commonly recorded independently of the time over which the effect lasted or area under the curve. Late toxic effects vary and tend to progress in time so that the peak effects are less clear than those in acute toxicity. Furthermore, whether this effect is reversible or correctable by medical or surgical measures or is permanent, progressive, and debilitating all weighs into the scale or grade chosen to clearly describe the late effect. A GTDH based on parameters of both the acute and late effects would provide a more accurate index for this area under the curve. If studies of longitudinal late effects were developed, such GTDH curves could be generated and organized through computerization of information. Conceivably, a formula could be developed employing the basics of late and acute toxicity scores, with provision for change over time; the Karnofsky status; and the number of organs involved. Such a formula, carefully derived, could provide clinically useful information.

Are there good laboratory models for late toxic effects?

Most of the laboratory and experimental systems that have been designed to study toxicity are more often concerned with acute rather than late toxic effects. Laboratory

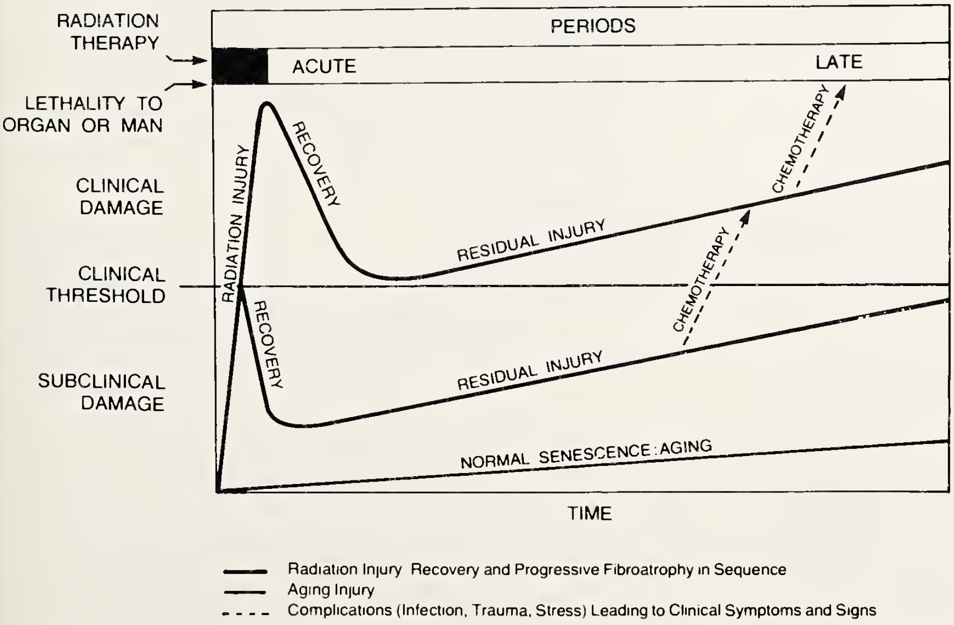


FIGURE 2.—Although normal tissues may have recovered from overt effects of radiation, salvage chemotherapy administered months to years later could trigger renewed toxic effects. This may be acute effect of chemotherapy or late effect of radiotherapy.

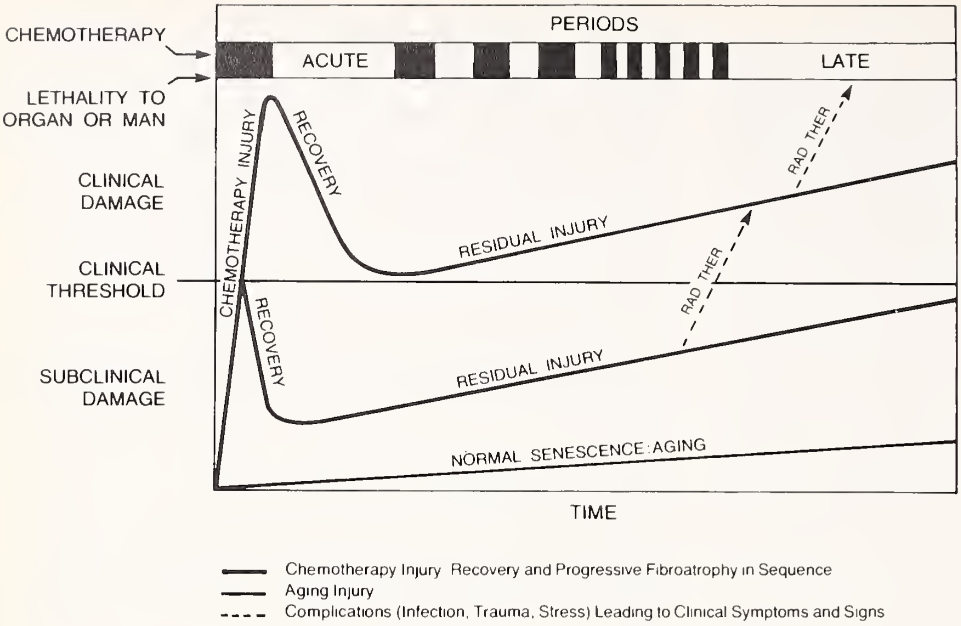


FIGURE 3.—Corollary to fig. 2 is case in which chemotherapy is "pulsed" in over extended period and radiotherapy is given after appropriate recovery break. Resulting toxicity may be considered acute due to radiotherapy or late due to chemotherapy.

modeling for late effects is more demanding, and additional systems need to be developed, with a shift from the cell-survival curves studied in vitro as well as in vivo to more complex pathophysiological or biochemical consequences of treatment. Again, such data must be reliable and reproducible. Most normal tissue models available for the study of drug-radiation reactions have been summarized by Field and Michalowski (*31*) and have been further modified and tabulated in this report. Review of the laboratory models that assess combined modality effects demonstrates that both acute and chronic toxic effects are occurring. A major problem in the thorough study of chronic toxic effects is the high cost of long-term laboratory studies, particularly when animals are used to simulate clinical situations. It has been stated that compared to tumors, normal tissue may be more consistent in the response to radiation since there is less heterogeneity. However, this is not always true. For example, in assessing the murine models for late toxic effects in lungs following irradiation, there is considerable variation among murine strains in regard to the dose and time required for development of pneumonitis and fibrosis. Explanations for this need to be defined and may relate to secondary factors such as the types of endogenous bacteria and viruses that exist and whether immunosuppression occurs after irradiation, leading to interstitial pneumonitis.

The search for predictive biochemical, metabolic, or physiological parameters, in terms of early events following irradiation and/or chemotherapy that predict for adverse effects, is a major direction of research and hopefully can be used to monitor the more permanent pathological damage.

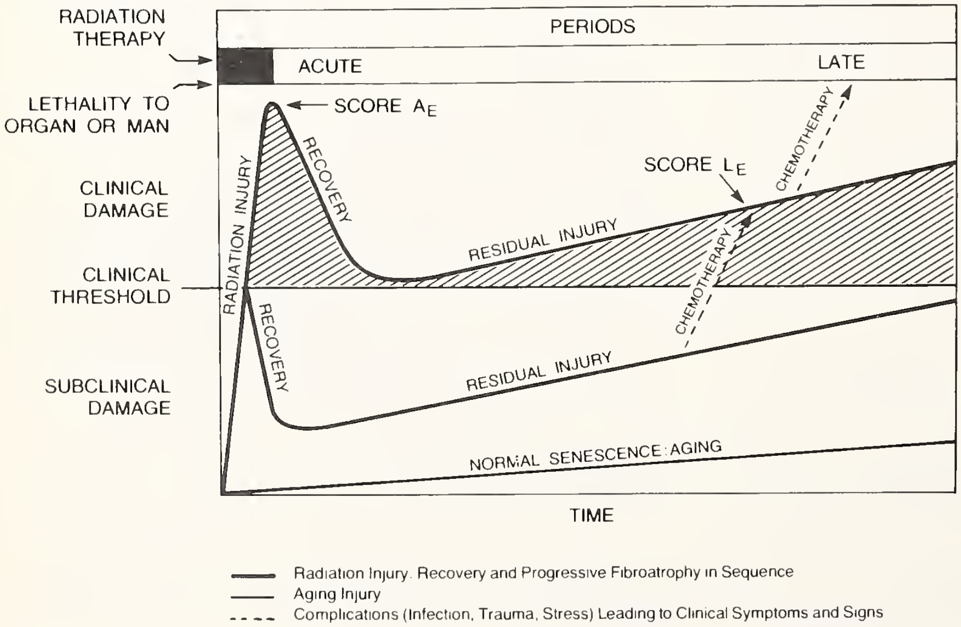


FIGURE 4.—This GTDH indicates peak score of acute toxic effects (A_E), while late effects (L_E) vary and tend to progress in time so that peak effect is less clearly defined.

CONCLUSIONS AND RECOMMENDATIONS

Following the toxicity conference held in Baltimore, MD, in September 1985, a subcommittee composed of representatives of several of the cooperative groups and the National Cancer Institute was formed to address the question of standardizing toxic effects criteria. The following is a summary of the conclusions and recommendations made by this committee.

1) There is a need for uniform toxicity scoring, both for acute and late toxic effects but particularly with regard to late effects in vital organs.
2) The toxicity scale should consist of five values, with emphasis on grade 3–5 levels and consideration of both peak grade and time in developing an appropriate index.
3) In addition to the major modalities of radiation therapy and chemotherapy, which are known to produce such late effects, the effects of host cofactors, other modalities, and recurrent tumor should be carefully considered.
4) End points should be clearly defined in terms of somatic injury, genetic alteration, and second malignant tumors, with appropriate time scales set, preferably >6 months following the introduction of treatment. There is need for a working committee on late effects by anatomic site; the committee should represent all major modalities, including a radiation oncologist, a medical oncologist, and a surgical oncologist, depending on the site.
5) Protocols should be developed for longitudinal studies of key dose-limiting normal tissues and organs, using the appropriate standard diagnostic laboratory and imaging tests. These should include CT, MRI, and positron emission tomography scans.
6) Actuarial risk reporting and follow-up of long-term survivors should be done initially in select patient cohorts; namely, in patients surviving >2 years. Although malignancies in which there is 50% survival have been considered most appropriate for such studies, this requirement may be too stringent; instead, the assessment of adverse effects should be done in all patients surviving >2 years. This is particularly true for highly fatal cancers, which are treated with intensive regimens of combined modalities.
7) Published studies should routinely present therapeutic ratios and use standard scoring for late toxic effects, as well as tumor response rates.

REFERENCES

(*1*) Rubin P: The Franz Buschke lecture: Late effects of chemotherapy and radiation therapy: A new hypothesis. Int J Radiat Oncol Biol Phys 10:5–34, 1984.

(*2*) Ash P: The influence of radiation on fertility in man. Br J Radiol 53:271–278, 1980.

(*3*) Sobrinho LG, Levine RA, DeConti RC: Amenorrhea in patients with Hodgkin's disease treated with antineoplastic agents. Am J Obstet Gynecol 109:135–139, 1971.

(*4*) Shalet SM, Morris-Jones PH, Beardwell CG, et al: Pituitary function after treatment of intracranial tumours in children. Lancet 2:104–107, 1975.

(*5*) Li FP: Adverse effects of treatment: Second cancers. *In* Cancer: Principles and Practice of Oncology (DeVita VT Jr, Hellman S, Rosenberg SA, eds), 2nd ed. Philadelphia: Lippincott, 1985, pp 2040–2043.

(*6*) D'Angio G (ed): Delayed consequences of cancer therapy: Proven and potential. Cancer 37:979–1236, 1976.

(*7*) Brady LW (ed): Long-term normal tissue effects of cancer treatment. Cancer Clin Trials 4(suppl 7):9–71, 1981.

(*8*) Rubin P: Radiation toxicology: Quantitative radiation pathology for predicting effects. Cancer 39(suppl 2): 729–736, 1977.

(*9*) Yarbro JW, Bornstein RS, Mastroangelo MJ: Seminars in oncology: Toxicity of chemotherapy. Semin Oncol 9:1–154, 1982.

(*10*) DeVita VT, Oliverio VT, Muggia FM, et al: The drug development program of the development of cancer treatment, NCI. Cancer Clin Trials 2:195–217, 1979.

(*11*) Kennedy BJ, Theologides A: The role of 5-fluorouracil in malignant disease. Ann Intern Med 55:719–730, 1961.

(*12*) Rubin P, Casarett GW: Clinical Radiation Pathology, vols I and II. Philadelphia: Saunders, 1968.

(*13*) Phillips TL: Tissue toxicity of radiation-drug interactions. *In* Radiation-drug Interactions in the Treatment of Cancer (Sokal GH, Maickel RP, eds). New York: Wiley, 1980, pp 175–200.

(*14*) Botnik LE, Hannon EC, Hellman S: Multisystem stem cell failure after apparent recovery from alkylating agents. Cancer Res 38:1942–1947, 1978.

(*15*) Von Hoff DD, Rozencweig M, Layard M, et al: Daunomycin-induced cardiotoxicity in children and adults: A review of 110 cases. Am J Med 62:200–208, 1977.

(*16*) Luna MA, Bedrossian CW, Lichtiger B: Interstitial pneumonitis associated with bleomycin therapy. Am J Clin Pathol 58:501–510, 1972.

(*17*) Slavin RE, Dias MA, Saral R: Cytosine arabinoside induced gastrointestinal toxic alterations in sequential chemotherapeutic protocols: A clinical-pathological study of 33 patients. Cancer 42:1747–1759, 1978.

(*18*) Fair WR: Urologic emergencies. *In* Cancer: Principles and Practice of Oncology (DeVita VT Jr, Hellman S, Rosenberg SA, eds), 2nd ed. Philadelphia: Lippincott, 1985, pp 1894–1905.

(*19*) Baum ES, Gaynon P, Greenberg L, et al: Phase II trial of cisplatin in refractory childhood cancer: Children's Cancer Study Group Report. Cancer Treat Rep 65:815–822, 1981.

(*20*) Rubin P: Position paper: Toxicity. International clinical trials in radiation oncology. Int J Radiat Oncol Biol Phys. In press.

(*21*) Deeg HJ, Storb R, Thomas ED: Bone marrow transplantation: A review of delayed complications. Br J Haematol 57:185–208, 1984.

(*22*) Creaven PJ, Mihich E: The clinical toxicity of anti-cancer drugs and its prediction. Semin Oncol 4:147–163, 1977.

(*23*) Kennedy BJ: Specific considerations for geriatric patients with cancer. *In* Medical Oncology (Calabresi P, Schein PS, Rosenberg SA, eds). New York: Macmillan, 1985, pp 1433–1445.

(*24*) Bonadonna G, Valagussa P: Chemotherapy of breast cancer: Current views and results. Int J Radiat Oncol Biol Phys 9:279–297, 1983.

(*25*) Von Hoff DD, Layard MW, Basa P, et al: Risk factors for doxorubicin-induced congestive heart failure. Ann Intern Med 91:710–717, 1979.

(*26*) Selker RG, Jacobs SA, Moore PB: BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) induced pulmonary fibrosis. Neurosurgery 7:560–565, 1980.

(*27*) Fajardo LF, Stewart JR: Pathogenesis of radiation-induced myocardial fibrosis. Lab Invest 29:244–257, 1973.

(*28*) Billingham ME: Endocardial changes in anthracycline-treated patients with and without irradiation. Front Radiat Ther Oncol 13:67–81, 1979.

(*29*) ELTRINGHAM JR: Cardiac response to combined modality therapy. Front Radiat Ther Oncol 13:161–174, 1979.

(*30*) GOORIN AM, BOROW KM, GOLDMAN A, et al: Congestive heart failure due to Adriamycin cardiotoxicity: Its natural history in children. Cancer 47:2810–2816, 1981.

(*31*) FIELD SB, MICHALOWSKI A: Endpoints for damage to normal tissues. Int J Radiat Oncol Biol Phys 5:1185–1196, 1979.

Interaction of Cisplatin and X-rays in Rat Kidney[1]

Eric van Rongen,[2,]* Willem C. Kuijpers,[2] and Albert J. van der Kogel[3,4]

ABSTRACT—Cisplatin was administered as a single iv dose of 5 mg/kg in WAG/Rij female rats at intervals of 7 days or 30 minutes before or 7 days after graded irradiation of the left kidney. The unirradiated right kidney was removed 4 weeks after the x-ray treatment. Kidney function was determined by measuring urine osmolality and plasma urea. The kidney function parameters did not change measurably in animals treated with cisplatin alone. Only differences in urine osmolality were observed between the groups that received combined treatment or irradiation only. Long-term renal fibrosis was assessed by measuring the hydroxyproline content. Significant increases in renal hydroxyproline content were observed in animals receiving treatment with cisplatin either 7 days before or 7 days after irradiation, compared with animals receiving irradiation alone.—NCI Monogr 6:19–22, 1988.

The drug CDDP is a firmly established chemotherapeutic agent used in the treatment of cancer. However, CDDP has a number of adverse side effects; nephrotoxicity is considered to be the most serious toxic effect (*1–3*). When patients are treated with a combination of chemotherapy and radiotherapy and the kidney is in the radiation field, the already high radiosensitivity of the kidney may be increased due to the effect of CDDP administration.

It has been demonstrated that CDDP influences the response to radiation in various cell lines in vitro (*4–6*) and in tumor systems (*7–10*) and normal tissues in vivo (*11–13*). The purpose of this study was to elucidate the influence of drug-radiation sequencing on renal toxicity in the rat.

MATERIALS AND METHODS

Animals

In all experiments, WAG/Rij female rats were used. Animals were 12–13 weeks of age at the time of irradiation or control CDDP treatment.

Irradiation

The left kidney was irradiated with single doses of 300-kV x-rays using a Philips-Müller x-ray generator operated at 10 mA. The dose rate was 3.4 Gy/minute with a half-value layer of 2.0 mm Cu. The animals were anesthetized with enflurane (Ethrane) during irradiation (*14*).

Irradiation was performed while the animal was positioned on its back on a lead plate 4 mm thick, which was fixed on top of the x-ray tube. The kidney was palpated, carefully manipulated in a lateral skin fold, and fixed in position over a circular cutout (28-mm diameter) in the lead plate. In this way, only the kidney and its overlying skin were irradiated. Details of the procedure will be published in a separate communication (van Rongen E, van der Kogel AJ, Durham SK, in preparation). Care was taken to prevent the ureter and renal artery and vein from being clamped shut.

Drug

The CDDP (Bristol-Myers, Weesp, The Netherlands) was injected via the tail vein while the animals were under slight ether anesthesia. A dose of 5 mg of CDDP/kg was used in all combined radiation-drug treatments. Control animals received CDDP only.

Surgery

The NX of the right kidney was performed at 4 weeks after irradiation in the animals given a combined treatment or irradiation only; at 4 weeks after CDDP administration in the group given CDDP without irradiation; and at 13 weeks of age in animals that had only NX.

Determination of Kidney Function

Urine was collected for 24 hours at regular time intervals. Urine osmolality served as an indicator of tubular function. Osmolality was measured using a Cryomatic osmometer (model 3C2; Advanced Instruments, Inc., Needham Heights, MA). A blood sample was taken by orbital puncture under ether anesthesia, using heparinized capillary tubes. The plasma urea content served as an indicator of glomerular function and was determined using the urea enzymatic colorimetric test kit (Boehringer Mannheim, Mannheim, Federal Republic of Germany). Absorbance at 550 nm was read by a spectrophotometer (Ultrospec 4050, LKB Biochrom, Ltd., Cambridge, United Kingdom).

Hydroxyproline Assay

The Formalin-fixed kidney sections were rinsed overnight in tap water to remove excess Formalin, blotted dry, and dried in a vacuum exsiccator. Hydrolysis was performed according to standard methods (*15,16*), and the hydrolysates were stored at −20° C.

The HP concentration was determined using the colorimetric method described by Woessner (*17*) as modified by

---

ABBREVIATIONS: CDDP = cisplatin; NX = nephrectomy; HP = hydroxyproline; ED_{50} = 50% isoeffect dose(s).

[1] Supported by the Koningin Wilhelmina Fonds, The Netherlands Cancer Foundation, grant No. RBI 83-6.

[2] Radiobiological Institute TNO, Rijswijk, The Netherlands.

[3] Institute for Radiotherapy, Academic Hospital, St. Radboud, Nijmegen, The Netherlands.

[4] We thank Dr. Stephen K. Durham for assistance in preparation of the manuscript.

* *Reprint requests:* Eric van Rongen, Ph.D., Radiobiological Institute TNO, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands.

Stegemann and Stalder (*18*). The procedures used were those described by Meistrich et al. (*16*). The absorbance was read at 550 nm using the Ultrospec spectrophotometer.

Data Analysis

Data were analyzed in two ways. First, the mean values for each kidney function parameter were calculated for the different treatment groups at every time point investigated. The disadvantage of this method is that when, in a treatment group, the response with regard to a certain parameter is heterogeneous and an animal from that group dies of kidney malfunction, the mean value may significantly alter toward "less effect" values. In such cases, mean values give a false indication of the reaction of the group as a whole.

To avoid this problem, determination of isoeffect doses was applied as a second method of analysis. The ED_{50} were calculated by probit analysis for the two functional parameters, using the number of animals exceeding an arbitrarily chosen threshold value. A level 1.8 times that of the mean control value was chosen as the threshold for plasma urea, and a level 0.54 times that of the mean control value was chosen as the threshold for urine osmolality.

Plasma urea values higher than the chosen isoeffect levels and osmolality values lower than the chosen isoeffect levels were always associated with irreversible, but not immediately lethal, kidney damage.

RESULTS

There was a temporary increase in plasma urea values between 10 and 30 weeks in the groups treated with a dose of 6 or 8 Gy (fig. 1A, 1B). The subsequent decline in urea values indicates that partial recovery had occurred. There was a rapid increase in urea values to toxic levels in animals that received doses of 10 or 12 Gy (fig. 1C, 1D). No significant differences in urea values were observed among the treatment groups. No change was observed in plasma urea values in animals given 5 mg of CDDP/kg alone (fig. 1C).

The mean urine osmolality values in animals given a combined treatment were generally lower than in animals given irradiation alone (fig. 2). The late-occurring decrease in osmolality in animals given CDDP alone (fig. 2C) was also observed in animals that had only NX.

No data are available for animals that received 12-Gy x-rays alone or animals that received the highest radiation doses in the groups given CDDP at 30 minutes before irradiation, since these animals died before the start of the observation period at 16 weeks after irradiation.

Control values for plasma urea, which were obtained in animals that received NX only, ranged between 40 and 60 mg/100 ml during the entire observation period. The level of 1.8 times the mean control plasma urea value, used in the determination of ED_{50}, corresponds to 100 mg/100 ml.

Control values for urine osmolality ranged between 1,500 and 3,000 mosmol/kg for up to about 40 weeks after NX and gradually declined thereafter. The chosen isoeffect level of 0.54 times the mean control osmolality value corresponds to 1,000 mosmol/kg for up to 42 weeks after NX. Thereafter, it decreased linearly to 580 mosmol/kg at 84 weeks after NX.

The ED_{50} values calculated for both parameters continuously decreased with time for all treatments (fig. 3). The isoeffect doses for plasma urea did not differ between animals receiving combined treatment or irradiation only (fig. 3A). Administration of CDDP plus irradiation resulted in lower ED_{50} values for urine osmolality (fig. 3B), which suggests enhanced tubular damage in the combined treatments. The osmolality data for the combined treatment of CDDP given 30 minutes before irradiation were too limited for probit analysis.

Increases in renal HP content at 73–86 weeks after

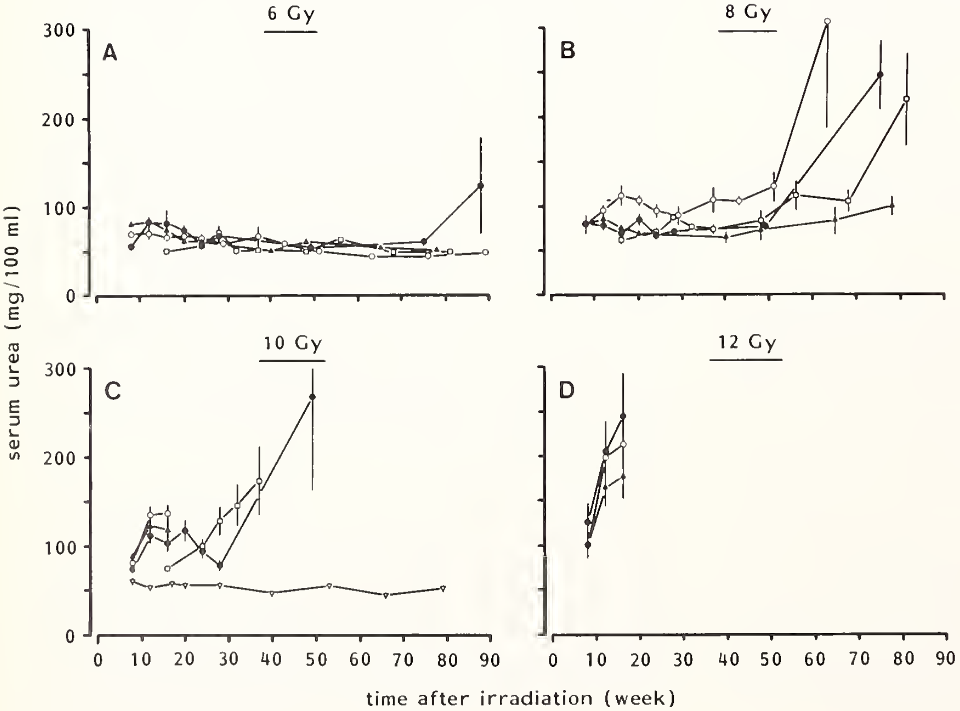


FIGURE 1.—Comparison per radiation dose of mean plasma urea values for different treatments. Means were plotted only when >2/3 of animals in group were still alive. *Bars* indicate SEM. A) x-ray dose = 6 Gy; B) x-ray dose = 8 Gy; C) x-ray dose = 9 Gy for irradiation alone and 10 Gy for combined treatments; D) x-ray dose = 12 Gy. □ = x-rays alone; ○ = CDDP given 7 days before x-rays; ▲ = CDDP given 30 min before x-rays; ● = x-rays given 7 days before CDDP; and ▽ = CDDP alone (C only).

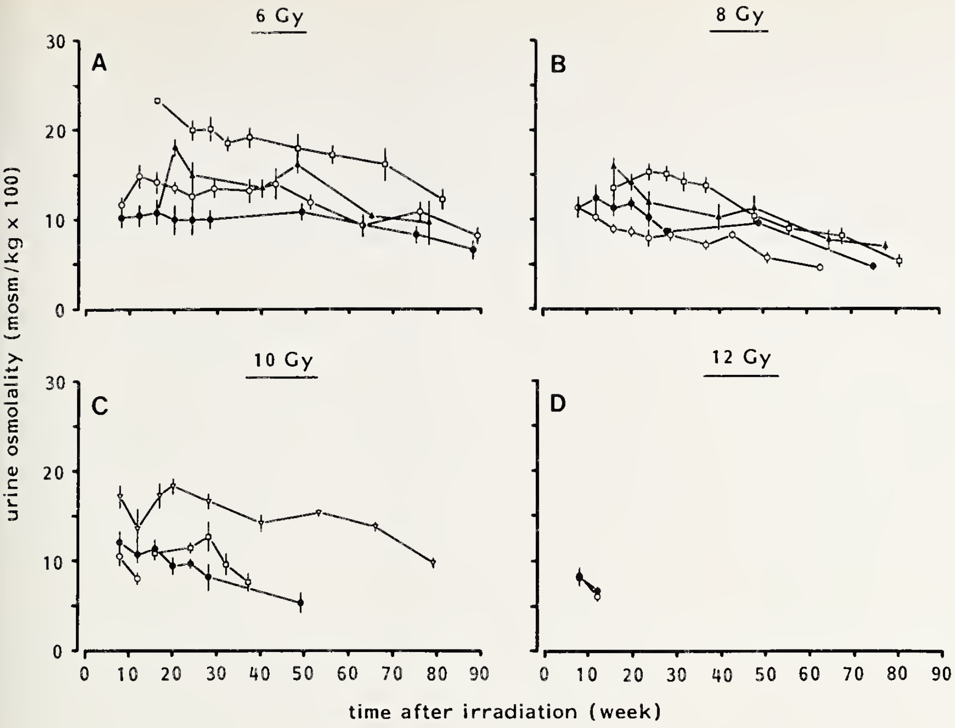


FIGURE 2.—Comparison per radiation dose of mean urine osmolality values for different treatments. For explanation of panels and symbols, *see* fig. 1 legend.

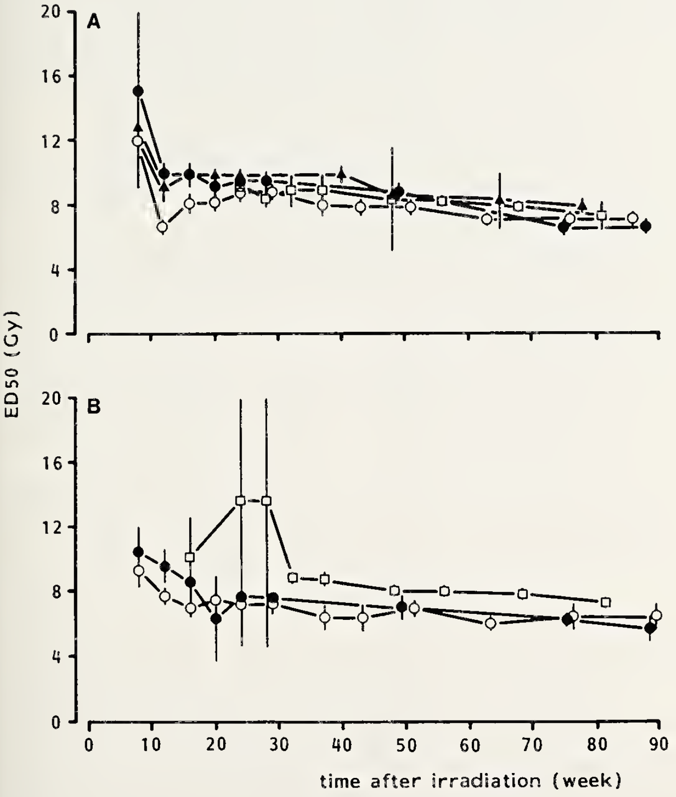


FIGURE 3.—ED_{50} calculated with probit analysis. *Bars* indicate SEM. A) isoeffect = plasma urea > 1.8 × mean control value; B) isoeffect = urine osmolality < 0.54 × mean control value. For explanation of symbols, *see* fig. 1 legend.

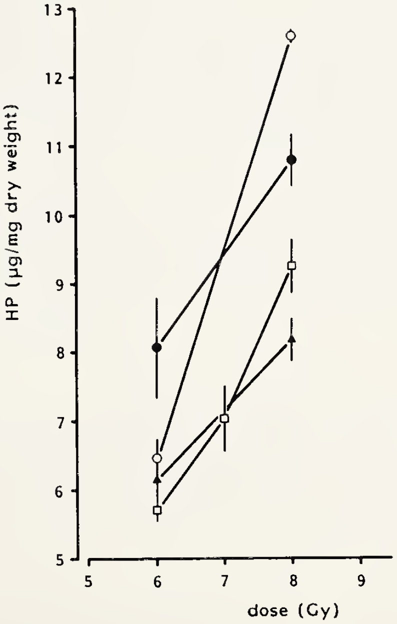


FIGURE 4.—HP content expressed per milligram of dry kidney weight. Only animals that died or were killed at 73–86 wk are included. Mean values and SEM are plotted. For explanation of symbols, *see* fig. 1 legend.

treatment were associated with increasing radiation dose (fig. 4). The HP content was higher in animals receiving CDDP at 7 days before or 7 days after irradiation, compared to rats given either CDDP 30 minutes before irradiation or irradiation alone.

DISCUSSION

The influence of the cytotoxic drug CDDP on the radioresponsiveness of cells in vitro and tumors and normal tissues in vivo is not clear. A number of different effects have been reported, including radiosensitization (*9,10,12*), repair inhibition (*4-6,11*), and independent action (*7,8*). The timing of CDDP administration with regard to irradiation is an important factor in determining the effectiveness of the combined treatment. The effect of CDDP on radiation-induced crypt cell depletion in the small intestine was greatest when CDDP was administered at 12 hours before to 6 hours after irradiation (*19*). The greatest CDDP-induced enhancement of radiation damage in the mouse kidney was observed when the drug was given at 0.5 hours before to 1 day after irradiation (*13*). However, it appeared that the overall dose-modifying effect was small, as dose-modifying factors of 1.1–1.3 were reported.

The results obtained in the present study are not suitable for a detailed analysis of the influence of CDDP on the renal response to x-rays because in the combined treatments a broad range of doses with increments of 2 Gy was chosen and a steep increase from minimal to full response was observed over a range of 7–9 Gy when irradiation was given alone. However, several general conclusions can be obtained from this study.

The agent CDDP has no measurable effect on the radiation-induced increases in plasma urea levels (fig. 1, 3A). Observed differences in urine osmolality values between combined treatments and irradiation only (fig. 2, 3B) may indicate that CDDP enhances damage to the tubular compartment.

The analysis of the HP content of the kidneys at 73–86 weeks demonstrates fibrosis to be more pronounced when CDDP is given 7 days before or 7 days after x-rays (fig. 4). This is reflected only in differences in the tubular function parameter, urine osmolality.

The results of this study indicate that CDDP, depending on the sequence of administration, may slightly enhance radiation-induced renal toxicity, with tubuli as the most likely target. Renal histopathological analysis (van Rongen E, van der Kogel AJ, and Durham SK, in preparation) will provide additional information, including the anatomic location of the fibrosis and the distribution of damage induced by the various treatment regimens.

Experiments now in progress in which CDDP and x-rays are given in a greater number of different sequences may provide a more detailed insight into the effect of CDDP administration on the response of the rat kidney to irradiation.

REFERENCES

(*1*) Gonzalez-Vitale JC, Hayes DM, Cvitkovic E, et al: The renal pathology in clinical trials of *cis*-platinum (II) diamminedichloride. Cancer 39:1362–1371, 1977.

(*2*) Dentino M, Luft FC, Yum MN, et al: Long term effect of *cis*-diamminedichloride platinum (CDDP) on renal function and structure in man. Cancer 41:1274–1281, 1978.

(*3*) Bitran JD, Desser RK, Billings AA, et al: Acute nephrotoxicity following *cis*-dichlorodiammine-platinum. Cancer 49:1784–1788, 1982.

(*4*) Dritschilo A, Piro AJ, Kelman AD: The effect of *cis*-platinum on the repair of radiation damage in plateau phase Chinese hamster (V79) cells. Int J Radiat Oncol Biol Phys 5:1345–1349, 1979.

(*5*) Carde P, Laval F: Effect of *cis*-dichlorodiammine platinum II and X rays on mammalian cell survival. Int J Radiat Oncol Biol Phys 7:929–933, 1981.

(*6*) Ziegler W, Trott K-R: Der effect einer combinierten Behandlung mit Cisplatin und Bestrahlung auf das Überleben von chinesischen Hamsterzellen. Strahlentherapie 161:308–315, 1985.

(*7*) Twentyman PR, Kallman RF, Brown JM: The effect of time between X-irradiation and chemotherapy on the growth of three solid mouse tumors. III. *cis*-Diamminedichloroplatinum. Int J Radiat Oncol Biol Phys 5: 1365–1367, 1979.

(*8*) Overgaard J, Kahn AR: Selective enhancement of radiation response in a C3H mammary carcinoma by cisplatin. Cancer Treat Rep 65:501–503, 1981.

(*9*) Douple EB, Richmond RC: Enhancement of potentiation of radiotherapy by platinum drugs in a mouse tumor. Int J Radiat Oncol Biol Phys 8:501–503, 1982.

(*10*) Lelieveld P, Scoles MA, Brown JM, et al: The effect of treatment in fractionated schedules with the combination of X-irradiation and six cytotoxic drugs on the RIF-1 tumor and normal mouse skin. Int J Radiat Oncol Biol Phys 11:111–121, 1985.

(*11*) Burholt DR, Schenken LL, Kovacs CJ, et al: Response of the murine gastrointestinal epithelium to *cis*-dichlorodiammine platinum II: Radiation combinations. Int J Radiat Oncol Biol Phys 5:1377–1381, 1979.

(*12*) von der Maase H: Effect of cancer chemotherapeutic drugs on the radiation-induced skin reactions in mouse feet. Br J Radiol 57:697–707, 1984.

(*13*) Stewart F, Bohlken S, Begg A, et al: Renal damage in mice after treatment with cisplatinum alone or in combination with X-irradiation. Int J Radiat Oncol Biol Phys 12:927–933, 1986.

(*14*) Ang KK, van der Kogel AJ, van der Schueren E: Inhalation anesthesia in experimental radiotherapy: A reliable and time-saving system for multifractionation studies in a clinical department. Int J Radiat Oncol Biol Phys 8:145–148, 1982.

(*15*) Law MP, Hornsey S, Field SB: Collagen content of lung of mice after X-irradiation. Radiat Res 65:60–70, 1976.

(*16*) Meistrich ML, Williams MV, Soranson J, et al: Increased collagen and fluid content of mouse kidneys at 9 months after single or fractionated X irradiation. Radiat Res 99:185–201, 1984.

(*17*) Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 93:440–447, 1961.

(*18*) Stegemann H, Stalder K: Determination of hydroxyproline. Clin Chim Acta 18:267–273, 1967.

(*19*) Dewit L, Oussoren Y, Bartelink H: Dose and time effects of *cis*-diamminedichloroplatinum (II) and radiation on mouse duodenal crypts. Radiother Oncol 4:363–371, 1985.

Renal Damage in Mice After Treatment With Cisplatin and X-rays: Comparison of Fractionated and Single-dose Studies [1]

F. A. Stewart,* A. Luts, Y. Oussoren, A. C. Begg, L. Dewit, and H. Bartelink [2,3]

ABSTRACT—Functional kidney damage in mice was measured after bilateral irradiation with x-rays alone or in combination with cisplatin (c-DDP). A single drug dose (6 mg/kg) was injected 30 minutes before the first of four or eight x-ray doses, given as four fractions per day with a minimum interval of 5 hours between treatments. A 30-fraction schedule was also investigated, with 15 fractions given in the first week (3 fractions per day), followed by a 2-week rest period and another 15 fractions in the fourth week. The c-DDP (4 mg/kg) was administered 30 minutes before the first fraction of each week, giving a total drug dose of 8 mg/kg. Renal function was assessed monthly from 10 to 37 weeks after the start of treatment by the clearance of ^{51}Cr-labeled EDTA. The combined treatment caused more kidney damage than either agent alone for all fractionation schedules. Enhancement of the radiation damage by c-DDP changed only slightly with fractionation; dose enhancement factors were 1.2 for 1 fraction to 1.3 for 30 fractions. Modeling studies showed that this was consistent with the additive toxic effects of the two agents. There was no change in the α/β for renal damage after x-rays plus c-DDP, compared with x-rays alone ($\alpha/\beta = 1.9$ Gy), implying that there was no reduction in repair and no modification of the x-ray response by c-DDP.—NCI Monogr 6:23-27, 1988.

It has been proved that c-DDP is one of the most effective chemotherapeutic agents for the treatment of certain types of cancer, including ovarian and testicular tumors (*1-3*). In addition to its cytotoxic properties, c-DDP has been shown to enhance radiation damage in several experimental tumor types (*4-7*), and clinical trials have now been initiated to evaluate the potential of c-DDP as a radiosensitizer (*8-11*). Some enhancement of normal tissue radiation damage has, however, also been demonstrated in intestine, esophagus, skin, and kidney (*12-18*). We have previously reported (*18*) that c-DDP given to mice before or after single doses of radiation caused a slight increase in functional renal impairment, with DEF values of 1.1-1.3, depending on the sequence and timing of the two agents.

One of the proposed mechanisms whereby c-DDP may increase radiation injury is by inhibition of repair (*19-23*). If this occurs in normal tissues as well as in tumors, a larger modification of x-ray damage can be expected in fractionated treatments than with single doses. The present study was undertaken to investigate the influence of c-DDP on renal radiation damage during fractionated treatments. The amount of repair of radiation damage that occurred after the combined treatment was compared with that after x-rays alone.

MATERIALS AND METHODS

Experimental Design

Fractionated irradiation was given in two ways. In one method, the schedule consisted of a range of x-ray doses given in four or eight fractions; there were four x-ray treatments per day, with a minimum interval of 5 hours between treatments and a total treatment time of 1-2 days. For these schedules, a single dose of 6 mg of c-DDP/kg ip (Platinol; Bristol-Myers Co.) was administered 30 minutes before the first x-ray dose. The overall treatment time was kept short to minimize any decrease in the effect of c-DDP with time.

In the other method, a range of total x-ray doses was given in 30 fractions (15 fractions per week); 3 fractions per day were given, with a minimum interval of 5 hours between treatments. After the first week, a 2-week rest period was allowed for repopulation of the small intestine, which was, unavoidably, partially included in the radiation field. In the fourth week, an additional 15 fractions were given as 3 fractions per day. The c-DDP was administered 30 minutes before the first fraction of each week, at a dose of 4 mg/kg (total dose, 8 mg/kg). The two drug schedules (one dose of 6 mg/kg or two doses of 4 mg/kg in 4 wk) were judged to be approximately equitoxic in terms of animal weight loss.

Irradiation

Female C3H inbred mice were used; they were 10-14 weeks of age and weighed 22-27 g at the start of the experiment. The irradiation procedures have been described in detail elsewhere (*18*). Kidneys of unanesthetized mice were bilaterally irradiated via lateral tangential fields (17 × 13 mm) with 250-kV x-rays (15 mA) filtered with 0.5 mm Cu. The dose rate at the position of the kidneys was 235 cGy/minute.

Measurement of Renal Function

Kidney function was measured from the clearance of ^{51}Cr-labeled EDTA (Amersham International). Measurements were made at monthly intervals from 10 to 37 weeks

---

ABBREVIATIONS: c-DDP = cisplatin; DEF = dose-enhancement factor; LQ = linear quadratic.

[1] Supported by the Koningin Wilhelmina Fonds, No. NKI 85-2, The Netherlands Cancer Foundation.

[2] Department of Experimental Radiotherapy, The Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Amsterdam.

[3] We thank Dr. J. Lebesque for criticism of the manuscript and Miss Thea Eggenhuizen for typing the manuscript.

* *Reprint requests:* F. A. Stewart, Ph.D., Department of Experimental Radiotherapy (H6), The Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

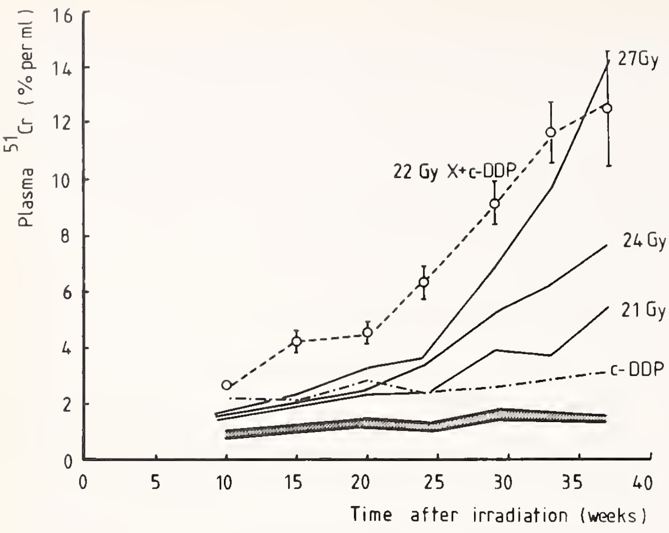


FIGURE 1.—Time course for changes in renal function after 4-fraction x-rays alone (——) or 4-fraction x-rays + c-DDP at dose of 6 mg/kg, 30 min before 1st fraction (○). Data points show mean values for 4–6 mice ±1 SEM. Renal function of untreated control mice is indicated by hatched area, and response after 6 mg of c-DDP/kg alone is also shown (— - —).

after the start of treatment. For clearance measurements, a single ip injection of ^{51}Cr-EDTA (10 μCi/mouse) was given and a single blood sample was taken from the retro-orbital sinus after 30 minutes (*18,24*). Blood samples were spun for 4 minutes at 12,000 *g*, and 15-μl samples of plasma were withdrawn and counted in an autogamma counter. Results were expressed as residual radioactivity (percent of injected activity) remaining in plasma at 30 minutes after injection of the tracer. This gives a quantitative estimate of renal clearance.

RESULTS

Some of the radiation treatments given over a short duration (1–2 days) were poorly tolerated due to damage to the small bowel. In particular, 3 dose groups were lost from the eight-fraction schedule of x-rays only, and as a result, this group has been excluded from these analyses. The 30-fraction schedules, using a 4-week treatment period, were much better tolerated, with no deaths due to acute intestinal damage.

Radiation-induced renal damage developed progressively from about 20 weeks after treatment. At earlier times, there was little renal impairment after x-rays alone, as has been previously reported (*18*). There was some renal impairment between 10 and 20 weeks after combined treatment, but this was not strongly related to x-ray dose. After 20 weeks, there was a dose-related, progressive deterioration in renal function, and the extent of damage was always more than that caused by x-rays alone. Figure 1 illustrates the time course for development of renal damage after four-fraction x-rays alone or in combination with c-DDP.

From 24 weeks after treatment, dose-response curves could be constructed, and the DEF for renal damage after x-rays alone could be compared with that after the combined treatment. For equivalent damage,

$$\mathrm{DEF} = \frac{\text{Dose of x-rays alone}}{\text{Dose of x-rays + c-DDP}}\ .$$

At earlier testing times, the dose-response curve for x-rays alone was too shallow to allow an accurate determination of DEF.

Figure 2 illustrates such dose-response curves at 33 weeks from the start of irradiation given in either 4 or 30 fractions, with and without c-DDP. There was a clear shift in the response toward lower x-ray doses after the combined treatment; DEF values (calculated at residual plasma activity levels of 6%–7%) were 1.26±0.04 for 4 fractions and 1.27±0.06 for 30 fractions. (Errors are ±1 SEM, calculated from fractional dose errors, using envelopes through the error bars on the dose-response curves.) The DEF, which was previously measured from single-dose studies at 33 weeks, was 1.18±0.08, with a slightly larger DEF (1.3) measured at the later testing time of 40 weeks (*18*). There was, therefore, little or no increase in DEF when c-DDP was given in combination with fractionated irradiation, compared with single doses.

To investigate the influence of c-DDP on the extent of repair of radiation injury, we considered the dose-response

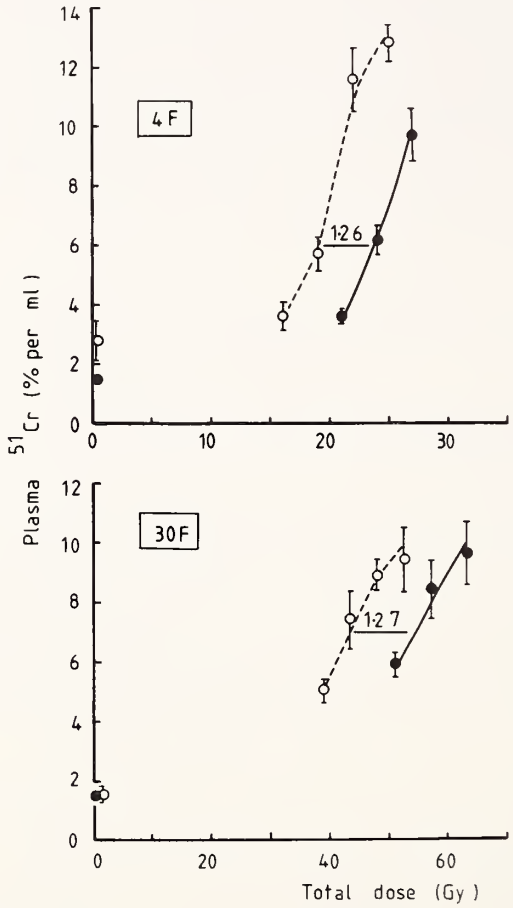


FIGURE 2.—Dose-response curves for renal damage 33 wk after start of irradiation schedules consisting of 4 or 30 fractions (F); ○ = x-rays + c-DDP (1 dose of 6 mg/kg + 4-fraction x-rays or 2 doses of 4 mg/kg + 30-fraction x-rays); ● = x-rays alone. Each data point represents mean value for groups of 4–6 mice ±1 SEM. DEF for c-DDP in each schedule is shown beside curves.

curves for treatment with 1–30 fractions of x-rays alone and 1–30 fractions of x-rays plus c-DDP (fig. 3). The single-dose curves are from reanalysis of data from a previously published experiment (*18*). There was clearly a great dose-sparing effect with fractionation after both x-rays alone and x-rays plus c-DDP. The dose increments from 1 to 30 fractions at an isoeffect level of 6% residual ^{51}Cr-EDTA activity were 36 ± 1.8 Gy for x-rays alone and 27.5 ± 1.6 Gy for x-rays plus c-DDP. (Errors are ±1 SEM, derived from envelopes of errors on the dose-response curves.) The dose recovered in fractionated treatments is, of course, dependent on the total dose (*25,26*). Since lower x-ray doses were used in all of the combined treatments because of the enhancement of response by c-DDP, the reduction in dose sparing after x-rays plus c-DDP cannot be interpreted as demonstrating reduced repair of radiation damage (*27*).

The data were analyzed in terms of an LQ model to determine α/β ratios for renal damage after x-rays plus c-DDP. Results from the combined treatment were compared with previously published data for 2, 5, 10, and 30 fractions of x-rays only (*28*). The concurrent data for x-rays alone were inadequate for an LQ analysis due to loss of the group that received 8 fractions, leaving only the 4- and 30-fraction groups. Figure 4 illustrates reciprocal total isoeffective doses for the different fractionation schedules, which are plotted as a function of dose per fraction. The data for x-rays only have previously been shown to give a good fit to an LQ model, with an α/β ratio of 1.9 ± 0.2 Gy at 30 weeks (*28*); this value was derived by multivariate regression analysis (±1 SEM). Although the present data for x-rays plus c-DDP are more limited, they also appear to be fitted by an LQ model, giving a straight line on a reciprocal total-dose plot (*29*). The α/β ratio was 1.7 ± 0.1 Gy (i.e., not significantly different from that for x-rays alone); this ratio was obtained by linear regression (±1 SEM).

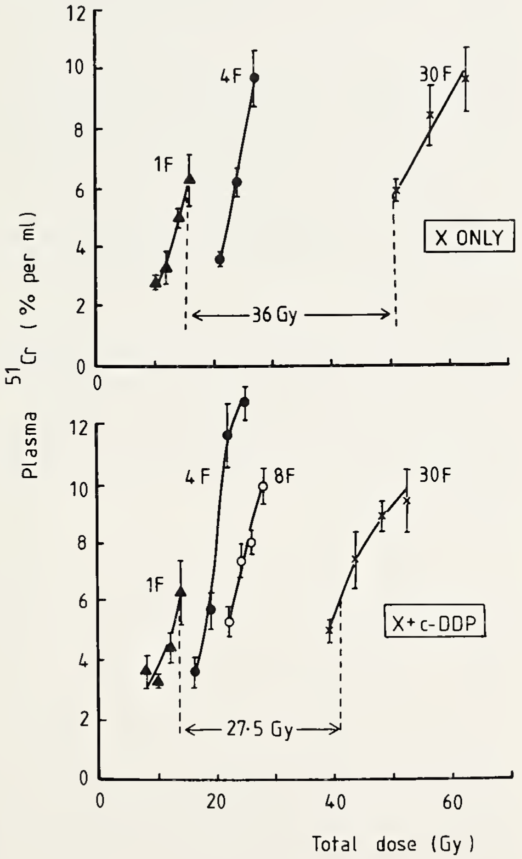


FIGURE 3.—Dose-response curves for renal damage 33 wk after 1–30 fractions of x-rays alone (*top*) and 1–30 fractions of x-rays + c-DDP (*bottom*). Each data point is mean value ±1 SEM for 4–6 mice. Recovered doses in mice receiving 30 fractions compared with single doses were 36 Gy for x-rays only and 27.5 Gy for x-rays + c-DDP. ▲ = reanalysis of data from previously published single-dose experiments (*18*); ● = 4 fractions; ○ = 8 fractions; x = 30 fractions.

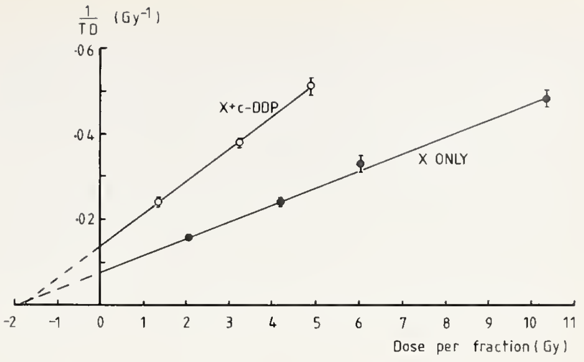


FIGURE 4.—Reciprocal total dose (TD) vs. dose/fraction for ^{51}Cr-EDTA clearance at 29–31 wk from midpoint of treatment. ○ = isoeffect doses calculated from dose-response curves for x-rays + c-DDP at level of 6% ^{51}Cr-EDTA activity. (Errors are ±1 SEM, obtained from envelope of errors.) ● = isoeffect doses (±1 SEM) from previously published experiments using treatment with 2–30 fractions of x-rays alone (*28*). Lines were fitted by linear regression, and back extrapolates on ordinate (---) indicate $-\alpha/\beta$ values.

DISCUSSION

Treatment with c-DDP was shown to increase renal damage when given in combination with fractionated radiation treatments. Increased damage was seen when a single drug dose (6 mg/kg) was given before the first of four or eight fractions in a total treatment time of 1–2 days. Enhanced damage was also seen when c-DDP was given in two doses of 4 mg/kg before the first and 16th fractions of a 30-fraction, 4-week schedule. The drug dose per injection was reduced to 4 mg/kg for the 30-fraction schedule, since previous toxicity studies had demonstrated that two doses of 4 mg/kg produced the same transient weight loss (1.2–1.5 g/mouse) as a single dose of 6 mg/kg. Very little functional renal impairment was, however, induced by two doses of 4 mg/kg alone, whereas 6 mg/kg produced a slight but persistent decrease in function (fig. 1).

The DEF measured from dose-response curves at 24–37 weeks after 4 and 30 fractions was 1.3. This compares with a previously measured DEF for single doses of 1.1–1.3, depending on the precise timing and sequence of the drug and x-rays (*18*). These DEF values include toxicity due to drug alone and probably largely reflect independent killing by x-rays and c-DDP. There was no increase in DEF with fractionation; an increase would be expected if c-DDP modified the sensitivity to radiation in the kidney or if it reduced the repair capacity.

A slight increase in the DEF for small intestine has been reported when c-DDP was given with fractionated irradiation, compared with that seen for single x-ray doses (*17*). This may indicate some inhibition of repair of x-ray damage by c-DDP in the intestine, as was reported by Luk et al. (*13*). It is, however, also consistent with a mainly additive response of the two agents (*17,27*).

Further support for the lack of any inhibition of x-ray repair by c-DDP in the kidney comes from the LQ analysis shown in figure 4. If c-DDP had modified the underlying survival curve with an associated reduction in repair, this would have been reflected in an increased α/β ratio (*27*). The α/β ratio derived for 4–30 fractions of x-rays plus c-DDP was, however, not significantly different from the α/β ratio for x-rays alone (1.7 ± 0.1 and 1.9 ± 0.2 Gy, respectively). The data used to derive an α/β ratio for the combination treatment included different overall treatment times (1 day to 4 wk) and different total drug doses (6–8 mg/kg). The LQ analysis assumes that changes in isoeffect with fractionation are the results of repair of radiation injury and that proliferation during treatment does not contribute. This is a reasonable assumption for the kidney for treatment times $\leq$4 weeks, since the cell turnover time is very long (*30,31*) and proliferation within 4 weeks of irradiation would not be expected and has not been demonstrated (*30-32*). It would, however, be desirable to have a constant overall treatment time for all fractionation schedules considered for an LQ analysis. Further experiments are therefore under way, extending the schedules for x-rays both alone and with c-DDP to enable a separate LQ analysis of fractionation data using the short treatment time of 2 days with a single drug dose of 6 mg/kg and the longer treatment time of 4 weeks with two doses of 4 mg of c-DDP/kg.

In conclusion, there was a small increase in the extent of renal radiation damage when c-DDP was given with single-dose or fractionated irradiation. The DEF was 1.2–1.3 and did not increase significantly with fractionation. There was no evidence for a reduction in repair of x-ray damage by c-DDP, and the observed enhancement of response was probably mainly due to additive toxic effects of the two agents.

REFERENCES

(*1*) Einhorn LH, Donohue JP: Improved chemotherapy in disseminated testicular cancer. J Urol 117:65–69, 1977.

(*2*) Ozols RF, Diesserothe AB, Javadpour N: Treatment of poor prognosis non-seminomatous testicular cancer with a “high-dose” platinum combination chemotherapy regimen. Cancer 51:1803–1807, 1983.

(*3*) Williams CJ, Head B, Arnold A, et al: Chemotherapy of advanced ovarian carcinoma: Initial experience using a platinum-based combination. Cancer 49:1778–1783, 1982.

(*4*) Bartelink H, Kallman RF, Rapacchietta D, et al: Therapeutic enhancement in mice by clinically relevant dose and fractionation schedules of *cis*-diamminedichloroplatinum (II) and irradiation. Radiother Oncol 6:61–74, 1986.

(*5*) Begg AC, Van der Kolk PJ, Dewit L, et al: Radiosensitization by cisplatin of RIF1 tumour cells in vitro. Int J Radiat Biol 50:871–884, 1986.

(*6*) Lelieveld P, Scoles MA, Brown JM, et al: The effect of treatment in fractionated schedules with the combination of X-irradiation and six cytostatic drugs on the RIF1 tumor and normal mouse skin. Int J Radiat Oncol Biol Phys 11:111–121, 1985.

(*7*) Overgaard J, Khan AR: Selective enhancement of radiation response in a C3H mammary carcinoma by cisplatin. Cancer Treat Rep 65:501–503, 1981.

(*8*) Reimer RR, Gahbauer R, Bukowski RM, et al: Simultaneous treatment with cisplatin and radiation therapy for advanced solid tumors: A pilot study. Cancer Treat Rep 65:219–222, 1981.

(*9*) Haselow RE, Adarus GS, Chen MM, et al: Simultaneous *cis*-platinum (DDP) and radiotherapy (RT) for locally advanced unresectable head and neck cancer. Int J Radiat Oncol Biol Phys 8:201, 1982.

(*10*) Shipley WU, Coombs LJ, Einstein AB, et al: *cis*-Platinum and full-dose irradiation for patients with invasive bladder carcinoma. Int J Radiat Oncol Biol Phys 9(suppl I):73, 1983.

(*11*) Dewit L, Bartelink H, Rümke P: Concurrent *cis*-diamminedichloroplatinum (II) and radiation treatment for melanoma metastases: A pilot study. Radiother Oncol 3:303–309, 1985.

(*12*) Phillips TL: Rationale for the selection of combined treatment schedules using fractionated radiation and chemotherapy. *In* Advances in Medical Oncology, Research and Education (Moore M, ed), vol VI. New York: Pergamon Press, 1979, pp 183–190.

(*13*) Luk KH, Ross GY, Phillips TL, et al: The interaction of radiation and *cis*-diamminedichloroplatinum (II) in intestinal crypt cells. Int J Radiat Oncol Biol Phys 5:1417–1420, 1979.

(*14*) Schenken LL, Burholt DR, Hagemann RF, et al: Combined modality oncotherapies. Front Radiat Ther Oncol 13:82–101, 1979.

(*15*) von der Maase H: Effect of cancer chemotherapeutic drugs on the radiation-induced skin reactions in mouse feet. Br J Radiol 57:697–707, 1984.

(*16*) von der Maase H: Interaction of radiation and Adriamycin, bleomycin, mitomycin C or *cis*-diamminedichloroplatinum (II) in intestinal crypt cells. Br J Cancer 49:779–786, 1984.

(*17*) Dewit L, Begg AC, Köhler Y, et al: Influence of *cis*-diamminedichloroplatinum (II) on mouse duodenal crypt stem cell survival after multifraction X-ray treatment. Int J Radiat Oncol Biol Phys 11:1809–1816, 1985.

(*18*) Stewart FA, Bohlken S, Begg AC, et al: Renal damage in mice after treatment with cisplatinum alone or in combination with X-irradiation. Int J Radiat Oncol Biol Phys 12:927–933, 1986.

(*19*) Alvarez MV, Cobreros G, Heras A, et al: Studies on *cis*-dichloroplatinum (II) as a radiosensitizer. Br J Cancer 37:68–72, 1978.

(*20*) Ziegler W, Locker B: Der Effekt einer Kombinierten Behandlung mit Cisplatin und Bestrahlung auf das Ueberleben von Nierenzellen des Syrischen Hamsters. Strahlentherapie 161:636–641, 1985.

(*21*) Dritschilo A, Piro AJ, Kelman AD: The effect of cis-platinum on the repair of radiation damage in plateau phase Chinese hamster (V-79) cells. Int J Radiat Oncol Biol Phys 5:1345–1349, 1979.

(*22*) Carde P, Laval F: Effect of *cis*-diamminedichloroplatinum (II) and X rays on mammalian cell survival. Int J Radiat Oncol Biol Phys 7:929–933, 1981.

(*23*) Towle LR, Hall EJ, Capuono L: Studies with *cis*-platinum diamminedichloride at the cellular level. Radiat Res 83:37–38, 1980.

(*24*) Williams MV, Denekamp J: Sequential functional testing of radiation induced renal damage in the mouse. Radiat Res 94:305–317, 1983.

(*25*) Travis EL, Parkins CS, Down JD, et al: Repair in mouse lung between multiple small doses of X-rays. Radiat Res 94:326–339, 1983.

(*26*) THAMES HD: Effect-independent measures of tissue response to fractionated irradiation. Int J Radiat Biol 45:1–10, 1984.

(*27*) BEGG AC: Additivity versus repair inhibition in fractionated treatments combining drugs and X rays: A theoretical analysis. Int J Radiat Biol Oncol Phys 13:921–927, 1987.

(*28*) STEWART FA, OUSSOREN Y, LUTS A, et al: Repair of sublethal radiation injury after multiple small doses in mouse kidney: An estimate of flexure dose. Int J Radiat Oncol Biol Phys 13:765–772, 1987.

(*29*) DOUGLAS BG, FOWLER JF: The effect of multiple small doses of X rays on skin reactions in the mouse and a basic interpretation. Radiat Res 66:401–426, 1976.

(*30*) GLATSTEIN E, BROWN RC, ZANELLI GD, et al: The uptake of rubidium-86 in mouse kidneys irradiated with fractionated doses of X rays. Radiat Res 61:417–426, 1975.

(*31*) SORANSON J, DENEKAMP J: Precipitation of latent renal radiation injury by unilateral nephrectomy. Br J Cancer 53(suppl VII):268–272, 1986.

(*32*) COLE LJ, ROSEN VJ: Latent radiation-inhibition and recovery of mitotic activity in mouse kidney. Exp Cell Res 23:416–418, 1961.

Effect of Total-body Irradiation With Bone Marrow Transplantation on Toxicity of Cisplatin[1]

John E. Moulder,[2,*] Brian L. Fish,[2] John S. Holcenberg,[3] and Mary Cheng[3]

ABSTRACT—In defined-flora, barrier-maintained rats (WAG/RijMCW males), radiation nephritis is the principal late toxicity seen after high-dose-rate, total-body irradiation (TBI) when hematologic toxicity is prevented by bone marrow transplantation. Pneumonitis develops only if rats are exposed to a conventional environment during and after bone marrow transplantation. Low-dose-rate TBI gives similar toxicity at doses twice as large. Rats surviving for 9 months after TBI show decreased tolerance for cisplatin. This decreased tolerance is related to dose and dose rate and is seen for radiation doses that show little or no renal toxicity. Evidence suggests that the decrease in renal tolerance is due to decreased renal platinum clearance in the irradiated kidneys.—NCI Monogr 6:29-33, 1988.

Bone marrow transplantation has an established role in the treatment of both malignant (*1-3*) and nonmalignant (*4*) disease. When bone marrow transplantation is used, the patient's marrow must be ablated, usually with TBI in combination with chemotherapy. With the hematopoietic toxicity of TBI prevented by marrow transplantation, the dose of TBI is limited by the tolerance of other tissues. High-dose TBI, alone or in combination with drugs, has been associated with toxicity to liver (*5,6*), lungs (*7-11*), gonads (*8*), and lens (*12*). In marrow transplant studies in rats, we (*13*) have reported an additional late tissue damage syndrome, which appears to be due to radiation nephritis. Similar renal toxicity after TBI and marrow transplant has been reported in mice (*9,14*), and recently it has been reported clinically (*15*).

The renal effects we have seen after TBI and bone marrow transplantation could affect the clearance and toxicity of chemotherapeutic agents. First, patients who relapse after TBI and bone marrow transplant appear to have low tolerance for phase I agents [(*16*); Holcenberg JS, unpublished observations]. Second, we have observed a decrease in cis-Pt and methotrexate clearance after low-dose renal irradiation in a rat model (*17*). Third, many of the agents used after bone marrow transplantation (e.g., cyclosporine and methotrexate) are nephrotoxic or are cleared through the kidneys (*18,19*). This report presents the results of studies of the cis-Pt tolerance of rats 9 months after TBI and bone marrow transplantation.

MATERIALS AND METHODS

Animals.—All studies were done with "defined microbiologically associated" male WAG/RijMCW rats housed in a "moderate security barrier" (*20*). Of particular importance to TBI studies, these rats are free of *Mycoplasma pulmonis, Pseudomonas,* and common rat viruses. All animals used in these studies were 2 months old at the start of the experiment. No antibiotic, immunosuppressive drugs, or any other known nephrotoxic agents (other than cis-Pt and irradiation) were used in these studies.

Total-body irradiation and bone marrow transplantation.—For high-dose-rate TBI, rats were irradiated without anesthesia by a posterior to anterior beam of 6-million electron volt x-rays at a dose rate of 64 cGy/minute. For low-dose-rate TBI, a posterior to anterior beam of cobalt-60 gamma rays was used at a dose rate of 3.25 cGy/minute. For both types of irradiation, a 3-cm Plexiglas block was placed in the beam to eliminate the skin sparing effect of these high energy beams. Dosimetry was done with an ion chamber in a Plexiglas phantom. The TBI dose was defined at the midline. The dose to the lung was 5%-10% higher than the midline dose; the dose to the remainder of the body was within 5% of the midline dose. Eighteen hours after TBI, rats were given an iv dose of 5×10^7 fresh syngeneic bone marrow cells according to published techniques (*21*).

Cisplatin.—The cis-Pt was supplied by the manufacturer (Bristol Laboratories, Syracuse, NY) and was used in the standard clinical formulation. Animals were hydrated prior to receiving the cis-Pt with an ip injection of 0.9% saline (3 ml/100 g). We gave the cis-Pt ip at a fixed time during the day to avoid diurnal variations in toxicity (*22*). We gave cis-Pt to all survivors of the high- and low-dose-rate TBI experiments 9 months after TBI, including control animals that had not received TBI or bone marrow transplantation. Animals were then placed in metabolic cages, and urine was collected over a 24-hour period. Total platinum in the urine was assayed by flameless atomic absorption spectroscopy (*23*).

Renal function testing.—At intervals after TBI, at 8 months after TBI (i.e., 1 mo prior to cis-Pt treatment), and 8 days after cis-Pt treatment (i.e., 9 mo and 8 days after TBI), blood and urine were collected for renal function

---

ABBREVIATIONS: TBI = total-body irradiation; cis-Pt = cisplatin; LD_{50} = lethal dose(s) for 50% of the animals.

---

[1] Supported by Public Health Service grant CA-24652 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Departments of Radiation Oncology and Pharmacology, Medical College of Wisconsin, Milwaukee.

[3] Departments of Pediatrics and Biochemistry (*J. S. Holcenberg*) and Department of Pathology (*M. Cheng*), University of Southern California, Los Angeles.

[*] *Reprint requests:* John E. Moulder, Ph.D., Department of Radiation Oncology, MCMC Box 165, Medical College of Wisconsin, 8700 W. Wisconsin Ave., Milwaukee, WI 53226.

testing. Urine creatinine, BUN, and urine protein were determined with the aid of commercial kits.

Statistical analysis.—We calculated the LD_{50} and compared them by probit analysis (*24*). Physiologic data were compared by two-tailed Mann-Whitney *U* tests and survival curves by an extension of the Kruskal-Wallis test (*25*). All values shown are means with 95% confidence intervals, and all significance testing was done at the .05 level.

RESULTS

Toxicity of Total-body Irradiation and Bone Marrow Transplantation

The acute (21-day) LD_{50} for high-dose-rate TBI was 6.9 (6.2–7.5) Gy in the absence of bone marrow transplantation and 13.2 (12.4–15.3) Gy when animals were given bone marrow transplantation (*13*). Animals that survived the acute lethality phase (which was due to gastrointestinal damage) showed a late lethality phase (fig. 1); the LD_{50} assessed at 8 months was 9.2 (9.0–9.4) Gy. These late deaths were accompanied by an increase in BUN and a decrease in urine creatinine levels (fig. 2), both indicators of radiation nephritis. No changes in breathing rate, an index of radiation pneumonitis, were observed after TBI when the animals were kept in a barrier environment (*13*). If animals were held in a conventional environment during and after TBI and bone marrow transplantation death occurred earlier, these deaths appeared to be due to pneumonitis (Moulder JE, unpublished observations).

The acute LD_{50} for low-dose-rate TBI was greater than 19 Gy when animals were given bone marrow transplantation. The late toxicity seen after low-dose-rate TBI (fig. 3) was similar to that after the high-dose rate; the doses to produce nephritis were about twice as high, with an 8-month LD_{50} of 17.1 (16.1–19.3) Gy. Figures 4 and 5 show urine creatinine levels 8 months after irradiation. A significant decrease in urine creatinine concentrations was seen at doses of 5.7 Gy and above in the high-dose-rate trials (fig. 4) and at doses of 13 Gy and above in the low-dose-rate trials (fig. 5). The BUN values (not shown) were abnormal at doses of 6.6 Gy and above in the high-dose-rate group and at doses of 15 Gy and above in those given the low-dose rate.

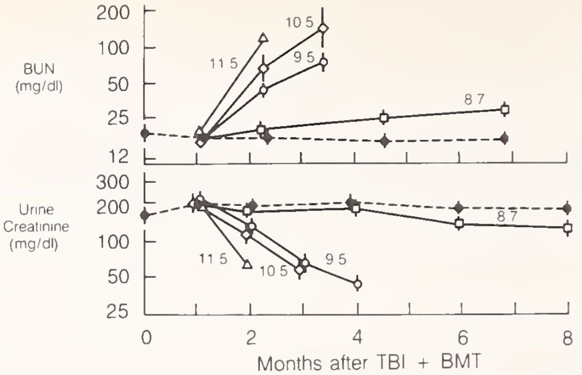


FIGURE 2.—Renal function after high-dose-rate TBI and bone marrow transplantation for the animals shown in fig. 1. Levels of BUN (*upper panel*) and urine creatinine (*lower panel*) are shown for control animals (●), and animals given 64 cGy/min TBI at doses of 8.7 (□), 9.5 (○), 10.5 (◇), and 11.5 Gy (△). Values shown are means with 95% confidence intervals, except for 11.5-Gy points at 2 mo, which are averages of 2 values.

Cisplatin Tolerance in Survivors of Total-body Irradiation

Figure 6 shows acute cis-Pt lethality dose–response curves for animals given cis-Pt 9 months after TBI. In animals that had not received TBI, the acute (13-day) LD_{50} for cis-Pt was 9.6 (9.0–10.6) mg/kg. In animals that had survived high-dose-rate TBI at doses of 5.7–7.5 Gy, the LD_{50} was 6.2 (5.5–7.1) mg/kg. In animals that had survived the low-dose rate at 11–15 Gy, the acute cis-Pt LD_{50} was 7.4 (6.0–9.2) mg/kg. The dose modification factor for the decrease in cis-Pt tolerance was 0.65 (0.57–0.75) for high- and 0.73 (0.61–0.90) for low-dose-rate TBI.

There were insufficient numbers of animals in the separate dose-rate groups for us to analyze the dependence of cis-Pt toxicity on the TBI dose. To analyze the dose dependence, we combined high- and low-dose-rate data on animals given TBI at doses near the 8-month LD_{50} (8.7–9.6 Gy high-dose rate and 15–19 Gy low-dose rate) and com-

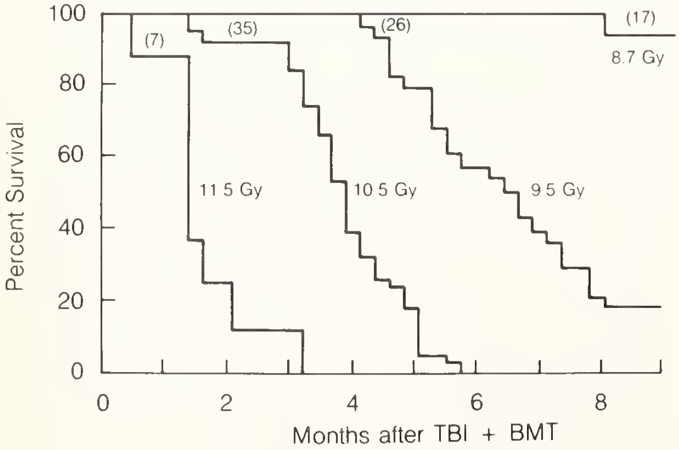


FIGURE 1.—Survival after high-dose-rate TBI (64 cGy/min) and bone marrow transplantation (BMT). The No. of animals at risk is shown *in parentheses.*

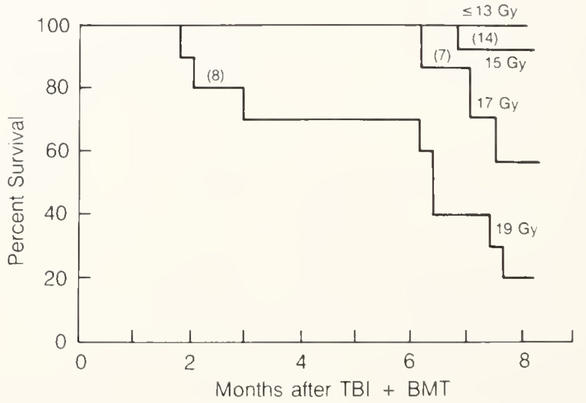


FIGURE 3.—Survival after low-dose-rate TBI (3.25 cGy/min) and bone marrow transplantation. The No. of animals at risk is shown *in parentheses.*

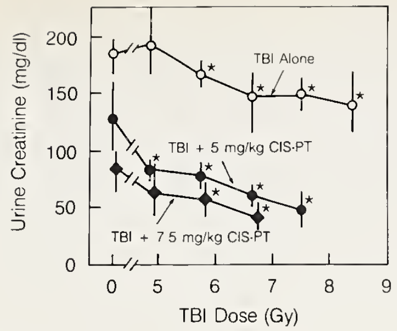


FIGURE 4.—Effect of cis-Pt on renal function in survivors of high-dose-rate TBI. A single dose of cis-Pt was given 9 mo after TBI. Urine creatinine levels (an index of renal function) are shown for irradiated animals 8 mo after TBI (○) and for the same animals 8 days after they received 5 (●) or 7.5 (◆) mg/kg of cis-Pt. For TBI alone, points are means of values for 15–20 animals; TBI plus cis-Pt points are means of values for 6–8 animals. All values are shown with 95% confidence intervals. *Asterisks* indicate urine creatinine levels that are significantly different from those of unirradiated animals that received the same cis-Pt dose.

pared them to animals given TBI at lower doses (4.9–7.6 Gy high-dose rate and 11–13 Gy low-dose rate). Both the high- and low-dose groups have significantly lower cis-Pt tolerance than do the unirradiated animals, with acute LD_{50} values of 6.2 (5.6–6.9) mg/dl and 7.5 (6.6–9.5) mg/kg, respectively.

Figures 4 and 5 show urine creatinine levels after cis-Pt treatment in the high- and low-dose-rate TBI survivors. After treatment with 5 mg cis-Pt/kg, irradiated animals had lower urine creatinine levels than did the unirradiated animals for TBI doses of 4.9 Gy and above in the high-dose-rate group (fig. 4) and for TBI doses of 13 Gy and above in the low-dose-rate group (fig. 5). Urine creatinine levels after treatment with 7.5 mg cis-Pt/kg showed similar results (fig. 4, 5).

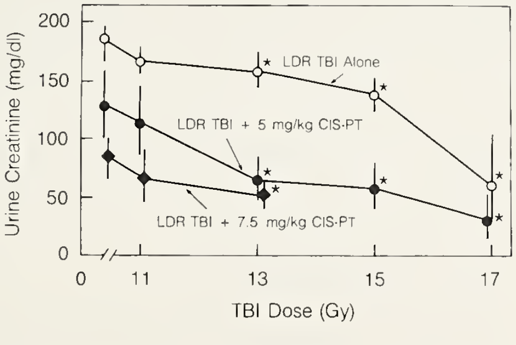


FIGURE 5.—Effect of cis-Pt on renal function in survivors of low-dose-rate (LDR) TBI. A single dose of cis-Pt was given 9 mo after TBI. Urine creatinine levels (an index of renal function) are shown for irradiated animals 8 mo after TBI (○), and for the same animals 8 days after they received 5 (●) or 7.5 (◆) mg/kg of cis-Pt. For TBI alone, points are means of values for 12–16 animals; for TBI plus cis-Pt, points are means of values for 5–7 animals. All values are shown with 95% confidence intervals. *Asterisks* indicate urine creatinine levels that are significantly different from those of unirradiated animals that received the same cis-Pt dose.

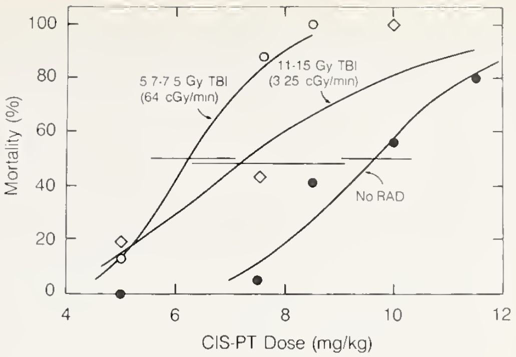


FIGURE 6.—Acute cis-Pt lethality in survivors of high- and low-dose-rate TBI and bone marrow transplantation. Percent of animals dying within 14 days of cis-Pt treatment are shown for control animals that did not receive TBI (●), for animals that received 5.7–7.5 Gy high-dose-rate TBI (○), and for animals that received 11–15 Gy low-dose-rate TBI (◇). There are 12–18 animals per cis-Pt dose in the high-dose-rate groups and 10–15 animals per cis-Pt dose in the low-dose-rate and cis-Pt alone groups. Curves are fitted by probit analysis (*24*), and the LD_{50} values are shown with 95% confidence intervals.

On an individual animal basis, urine creatinine levels 8 months after treatment were not predictive of cis-Pt tolerance. The normal urine creatinine level in 10-month-old male rats was 184 (175–195) mg/dl. Animals with urine creatinine levels of 160 mg/dl and above 8 months after TBI had a cis-Pt LD_{50} of 7.0 (6.0–8.9) mg/dl, whereas animals with urine creatinine levels below 160 mg/dl had a cis-Pt LD_{50} of 6.7 (6.0–7.5) mg/dl. A similar division of the survivors on the basis of BUN also showed no difference in cis-Pt tolerance, but the analysis was weakened by the relatively small number of survivors with abnormal BUN values.

Cisplatin Clearance in Survivors of Total-body Irradiation

The rate of urinary excretion of platinum is an index of the systemic exposure to cis-Pt, with decreased clearance generally associated with increased toxicity (*17*). Table 1 shows the 24-hour clearance of platinum in the TBI survivors. At doses of 7.6 Gy and above in the high-dose-rate group and 15 Gy and above in the low-dose-rate group,

TABLE 1.—Platinum clearance in TBI survivors[a]

TBI			Platinum cleared in 24 hr[b]	
Dose, Gy	Dose rate, cGy/min	No. assayed	%	Confidence interval
No TBI	—	32	34.5	30.1–40.5
4.9–5.8	64	8	39.7	26.2–60.2
6.7–7.6	64	17	28.4	22.3–36.1[c]
8.7–9.6	64	6	25.4	17.6–36.7[c]
11–13	3.25	14	35.7	25.9–49.2
15–17	3.25	12	18.2	13.5–24.7[c]

[a] Clearance was assessed 9 mo after TBI.

[b] Values are percent injected platinum cleared into the urine within 24 hr of cis-Pt injection (mean with 95% confidence interval).

[c] Clearance was significantly ($P<.05$ by Mann-Whitney U test) less than that of animals not receiving TBI.

platinum excretion is significantly decreased compared with that seen in unirradiated age-matched controls. Survivors of lower doses of TBI show normal excretion of platinum.

DISCUSSION

It has long been speculated that radiation damage to organs might affect the pharmacokinetics, and hence the toxicity, of subsequently administered drugs (*26–30*). We (*17*) have shown that renal irradiation can affect the renal clearance of cis-Pt and methotrexate. We have now found that animals that survive TBI and bone marrow transplantation have decreased tolerance for cis-Pt. The cis-Pt tolerance dose in survivors is 60%–75% that of unirradiated age-matched animals, with the amount of the decrease depending on the dose (fig. 7) and dose rate (fig. 6) of TBI. The decrease in tolerance can be detected at doses which show little or no evidence of renal dysfunction (fig. 4, 5). Thus cis-Pt tolerance appears to be a more sensitive indicator of renal damage than our renal function tests.

In previous work, we (*17*) have shown that bilateral kidney irradiation decreases cis-Pt tolerance. After a course of fractionated renal irradiation (20 Gy in 9 fractions) that produces only 6% mortality at 9 months, there is a progressive decrease in cis-Pt tolerance beginning at 3 months. This decrease in drug tolerance is paralleled by a decrease in urine platinum excretion. Because the rate of urine platinum excretion is an index of systemic exposure to cis-Pt, we have proposed that the decrease in cis-Pt tolerance seen after renal irradiation is due to increased cis-Pt exposure caused by decreased platinum excretion in the radiation-damaged kidneys (*17*). We have now demonstrated that the decrease in cis-Pt tolerance after TBI and bone marrow transplantation is also related to decreased platinum clearance. If decreased drug clearance is the mechanism for the decrease in cis-Pt tolerance, then decreased drug tolerance would be expected after TBI for all renally cleared drugs, not just for drugs with renal toxicity.

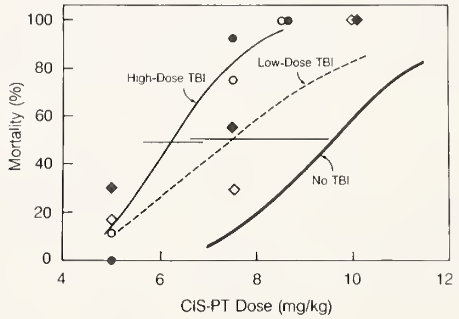


FIGURE 7.—Acute cis-Pt lethality in high- and low-dose survivors of TBI and bone marrow transplantation. Percent of animals dying within 14 days of cis-Pt treatment are shown for animals that received high-dose-rate TBI at 8.7–9.6 Gy (●), and 4.9–7.6 Gy (○); animals that received low-dose-rate TBI at 15–19 Gy (◆) and 11–13 Gy (◇). There were 5–10 animals per cis-Pt dose in the high-dose-rate groups and 4–7 animals per cis-Pt dose in the low-dose-rate groups. Probit curves (*24*) were fit separately to the high-dose (●, ◆) and low-dose (○, ◇) data, and the probit curve for control animals from fig. 6 is shown as a *heavy solid line*. The LD_{50} values are shown with 95% confidence intervals.

REFERENCES

(*1*) Thomas ED: The role of marrow transplantation in the eradication of malignant disease. Cancer 49:1963–1969, 1982.

(*2*) Appelbaum FR, Thomas ED: Treatment of acute leukemia in adults with chemoradiotherapy and bone marrow transplantation. Cancer 55:2202–2209, 1985.

(*3*) Helson L: Autologous bone marrow transplantation. Am J Pediatr Hematol Oncol 7:45–50, 1985.

(*4*) Bortin MM, Gale RP, Rimm AA: Allogeneic bone marrow transplantation for 144 patients with severe aplastic anemia. JAMA 245:1132–1139, 1981.

(*5*) McDonald GB, Sharma P, Matthews DE, et al: The clinical course of 53 patients with veno-occlusive disease of the liver after marrow transplantation. Transplantation 39:603–608, 1985.

(*6*) Deeg HJ, Storb R, Weiden PL, et al: High-dose total-body irradiation and autologous bone marrow reconstitution in dogs: Dose-rate related acute toxicity and fractionation-dependent long-term survival. Radiat Res 88:385–391, 1981.

(*7*) Bortin MM, Gale RP, Kay HEM, et al: Bone marrow transplantation for acute myelogenous leukemia: Factors associated with early mortality. JAMA 249:1166–1175, 1983.

(*8*) Deeg HJ, Storb R, Thomas ED: Bone marrow transplantation: A review of delayed complications. Br J Hematol 57:185–208, 1984.

(*9*) Travis EL, Peters LJ, McNeill J, et al: Effect of dose-rate on total body irradiation: Lethality and pathologic findings. Radiother Oncol 4:341–351, 1985.

(*10*) Keane TJ, VanDyk J, Rider WD: Idiopathic interstitial pneumonia following bone marrow transplantation: The relationship with total body irradiation. Int J Radiat Oncol Biol Phys 7:1365–1370, 1981.

(*11*) Depledge MH, Barrett A: Dose-rate dependence of lung damage after total body irradiation in mice. Int J Radiat Biol 41:325–334, 1982.

(*12*) Deeg HJ, Flournoy N, Sullivan KM, et al: Cataracts after total body irradiation and marrow transplantation: A sparing effect of dose fractionation. Int J Radiat Oncol Biol Phys 10:957–964, 1984.

(*13*) Moulder JE, Fish BL, Abrams RA: Renal toxicity following total body irradiation and syngeneic bone marrow transplantation. Transplantation 43:589–592, 1987.

(*14*) Covelli V, Metalli P, Briganti G, et al: Late somatic effects in syngeneic radiation chimaeras. II. Mortality and rate of specific diseases. Int J Radiat Biol 26:1–15, 1974.

(*15*) Bergstein J, Andreoli SP, Provisor AJ, et al: Radiation nephritis following total-body irradiation and cyclophosphamide in preparation for bone marrow transplantation. Transplantation 41:63–66, 1986.

(*16*) Poplack DG, Sallan SE, Rivera G, et al: Phase I study of 2′-deoxycoformycin in acute lymphoblastic leukemia. Cancer Res 41:3343–3346, 1981.

(*17*) Moulder JE, Holcenberg JS, Kamen BA, et al: Renal irradiation and the pharmacology and toxicity of methotrexate and cisplatinum. Int J Radiat Oncol Biol Phys 12:1415–1418, 1986.

(*18*) Loughran TP, Deeg HJ, Dahlberg S, et al: Incidence of hypertension after marrow transplantation among 112 patients randomized to either cyclosporine or methotrexate as graft-versus-host disease prophylaxis. Br J Hematol 59:547–553, 1985.

(*19*) Weiss RB, Poster DS: The renal toxicity of cancer chemotherapeutic agents. Cancer Treat Rev 9:37–56, 1982.

(*20*) Jonas AM, Desch EL, Brennan PC, et al: Long-term holding of laboratory rodents. Inst Lab Animal Res News 29:1–26, 1976.

(*21*) Abrams RA, Buck P, Fish BL, et al: Relationship of cell dose and freezing rate to reconstitutive potency in a hematopoietic reconstitution model. Transplantation 43:581–583, 1987.

(*22*) Hrushesky WJM, Levi FA, Halberg F, et al: Circadian stage dependence of *cis*-diamminedichloroplatinum lethal toxicity in rats. Cancer Res 42:945–949, 1982.

(*23*) Pera MF, Harder HC: Analysis for platinum in biological material by flameless atomic absorption spectroscopy. Clin Chem 23:1245–1249, 1977.

(*24*) Finney DJ: Statistical Methods in Biological Assay. New York: Hafner, 1964.

(*25*) Lee ET, Desu MM: A computer program for comparing K samples with right-censored data. Comput Methods Programs Biomed 2:315–321, 1972.

(*26*) Brown JM: Drug or radiation changes to the host which could affect the outcome of combined modality therapy. Int J Radiat Oncol Biol Phys 5:1151–1163, 1979.

(*27*) Rubin P: Late effects of chemotherapy and radiation therapy: A new hypothesis. Int J Radiat Oncol Biol Phys 10:5–34, 1984.

(*28*) Holcenberg JS, Kun LE, Ring BJ, et al: Effect of hepatic irradiation on the toxicity and pharmacokinetics of adriamycin in children. Int J Radiat Oncol Biol Phys 7:953–956, 1981.

(*29*) Griffin TW, Rasey JS, Bleyer WA: The effect of photon irradiation on blood–brain barrier permeability to methotrexate in mice. Cancer 40:1109–1111, 1977.

(*30*) Kamen BA, Moulder JE, Kun LE, et al: Effects of single-dose and fractionated cranial irradiation on rat brain accumulation of methotrexate. Cancer Res 44:5092–5094, 1984.

Effect of Sequencing on Combined Toxicity of Renal Irradiation and Cisplatin[1]

John E. Moulder* and Brian L. Fish[2]

ABSTRACT—We are using a rat model to study the effects of sequencing on the combined toxicity of renal irradiation and cisplatin (cis-Pt). Unanesthetized female WAG/RijMCW rats were given bilateral kidney irradiation (20 Gy in 9 fractions), preceded or followed by single ip doses of cis-Pt. Renal irradiation causes an increase in the acute toxicity of cis-Pt given 3–9 months after irradiation. Low-dose cis-Pt given immediately before irradiation, or as long as 9 months after irradiation, causes a decrease in the latent period for radiation nephritis and an increase in its severity. When given 3.7 to 7.7 months prior to irradiation, cis-Pt has a less severe effect on radiation nephritis. The greatest enhancement of radiation nephritis is seen for cis-Pt given 3 months after irradiation. Additive effects of cis-Pt and radiation on renal function can explain much, but not all, of the combined toxicity.—NCI Monogr 6:35–39, 1988.

Irradiation and cis-Pt are both nephrotoxic, although the nature, time of occurrence, and progression of the nephropathy caused by the two agents are different (*1–5*). Situations arise clinically when cis-Pt and renal irradiation are combined intentionally, or when one agent is used to treat recurrences after the other. For example, both renal irradiation and cis-Pt are used in the primary treatment of genitourinary cancer (*1,2*) and neuroblastoma (*6*). Combinations of radiation and cis-Pt may become more common, as there is in vitro and in vivo evidence that cis-Pt is a radiosensitizer (*7–9*).

Rodents are an appropriate model for the study of both radiation nephritis (*4,5,10*) and cis-Pt nephropathy (*4,5,11,12*). In a previous study in rats (*5*), we have shown that the acute toxicity of cis-Pt is the same in irradiated and unirradiated rats when cis-Pt is given immediately after renal irradiation. Beginning 3 months after irradiation, however, there is a progressive increase in cis-Pt toxicity and a simultaneous decrease in urinary platinum excretion, which indicates a pharmacokinetic mechanism for the increased acute toxicity. Stewart et al. (*4*) have shown in mice that cis-Pt given immediately before renal irradiation, or up to 1 week after irradiation, increases the severity of radiation nephritis. In the studies reported here, we investigated the effects of cis-Pt on the development of radiation nephritis when the cis-Pt is given months before or after fractionated renal irradiation.

MATERIALS AND METHODS

Animals.—All studies were done with "defined microbiologically associated" female WAG/RijMCW rats housed in a "moderate security barrier" (*13*). The animals were 2 months old at the start of the experiment. Rats were irradiated without anesthesia with 250-kV x-rays (half-value layer of 0.5 mm copper) and Plexiglas jigs adapted from those used by Sheldon et al. (*14*). Parallel-opposed lateral fields were used that encompassed both kidneys with a 5-mm margin. Animals were treated with 20 Gy in 9 fractions over 11 days, at a dose rate of 1.3 Gy/min.

Cisplatin.—The cis-Pt was supplied by the manufacturer (Bristol Laboratories, Syracuse, NY) and was used in the standard clinical formulation. Animals were hydrated prior to receiving cis-Pt with an ip injection of 0.9% saline (3 ml/100 g). We injected the cis-Pt ip at a fixed time during the day to avoid diurnal variations in toxicity (*15*).

Renal function testing.—Prior to the first treatment (irradiation or cis-Pt), 8 days after cis-Pt and 4 and 7 months after renal irradiation, blood and urine were collected for renal function testing. Urine and serum creatinine, BUN, and urine protein were determined with the aid of commercial kits. *N*-Acetyl-glucosamine was assayed according to published methods (*16*).

Statistical analysis.—We calculated the LD_{50} and compared them by probit analysis (*17*). Physiologic data were compared by two-tailed Mann-Whitney *U* tests. Survival curves were compared by an extension of the Kruskal-Wallis test (*18*). All values shown are means with 95% confidence intervals and all significance testing was done at the 0.05 level.

RESULTS

Nephrotoxicity of Fractionated Renal Irradiation Alone

The radiation schedule used in these experiments (9× 2.22 Gy) produced a chronic nephritis, the symptoms of which indicate progressive tubular damage. No deaths occurred in irradiated rats until 6 months after irradiation (fig. 1). Between 6 and 11 months, 6% of the irradiated animals died, and between 11 and 16 months an additional 27% died (fig. 1). A 20% higher radiation dose produced severe radiation nephritis at 4–9 months; a 20% lower dose produced no appreciable mortality until 15 months (fig. 1). In the control group, 1 rat died at 12 months, immediately

---

ABBREVIATIONS: cis-Pt = cisplatin; LD_{50} = lethal dose(s) for 50% of the animals.

---

[1] Supported by Public Health Service grant CA-24652 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Departments of Radiation Oncology and Pharmacology, Medical College of Wisconsin, Milwaukee.

* *Reprint requests:* John E. Moulder, Ph.D., Department of Radiation Oncology, MCMC Box 165, Medical College of Wisconsin, 8700 W. Wisconsin Ave., Milwaukee, WI 53226.

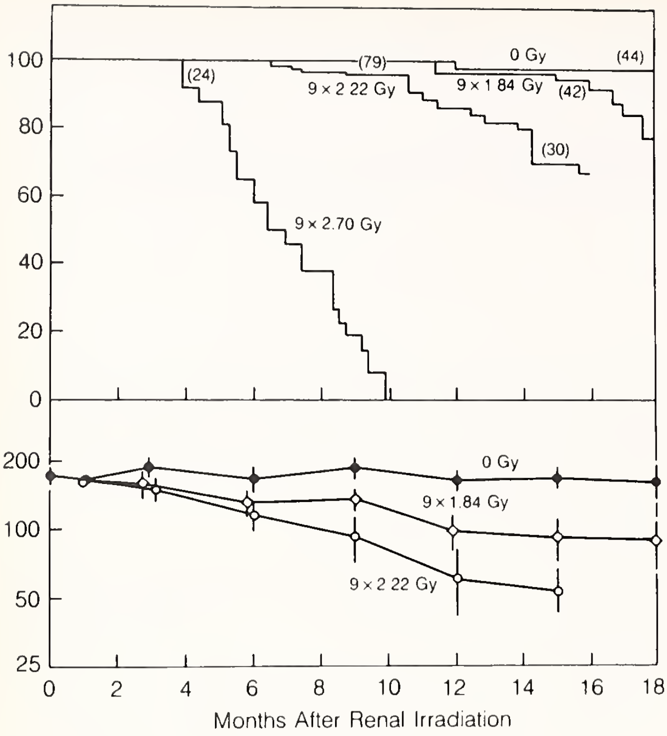


FIGURE 1.—Effects of fractionated renal irradiation on survival and renal function. *Upper panel:* Survival (percent) in irradiated and control animals. Data are shown for the radiation dose used in these experiments (9×2.22 Gy) plus higher and lower doses. The No. of animals at risk is shown *in parentheses. Lower panel:* Urine creatinine levels (milligrams/deciliter) in control animals (●), in animals treated at the dose used in these experiments (○), and animals treated at a lower dose (◇). Data are shown as geometric means with 95% confidence intervals.

after renal function testing; the cause of death could not be determined.

Urine creatinine levels were unchanged in control animals during the course of the experiment (fig. 1), as were BUN, urine volumes, urine protein levels, and *N*-acetyl-glucosamine to creatinine ratios (*5*). In irradiated animals, there was a progressive decrease in urine creatinine concentration beginning at 3 months (fig. 1). In irradiated rats, BUN, urine protein levels, *N*-acetyl-glucosamine to creatinine ratios, and blood pressure increased 3 months after irradiation and remained high for the course of the experiment (*5*). No animals that received fractionated renal irradiation alone died within 4 months of treatment, and all that died showed elevated BUN levels (>40 mg/dl) and decreased urine creatinine levels (<100 mg/dl) prior to death. Thus there was no evidence for deaths due to acute or late gastrointestinal toxicity.

Nephrotoxicity of Cisplatin Alone

The cis-Pt produced acute nephrotoxicity, with most deaths occurring within 10 days (fig. 2). Mortality was occasionally seen as late as 2 months after cis-Pt treatment. The LD_{50} for cis-Pt was 10.6 (10.1–11.2) mg/kg in 2-month-old rats and 9.5 (8.8–10.2) mg/kg in 12-month-old rats; the difference was small but statistically significant. Urine creatinine levels showed an immediate (within 1 wk) decrease after cis-Pt treatment, with little evidence for either recovery or progressive damage (fig. 2). This decrease in urine creatinine was paralleled by increases in BUN (not shown).

Nephrotoxicity of Renal Irradiation Followed by Cisplatin

One week and 3, 6.5, and 9.5 months after renal irradiation, animals were given single ip injections of cis-Pt at 5, 7.5, and 10 mg/kg. The LD_5 dose of cis-Pt (7.5 mg/kg) produced no acute lethality when given 1 week after irradiation, but it shortened the latent period for fatal radiation nephritis and greatly increased its severity (fig. 3). When 7.5 mg/kg was given at longer times after irradiation, it produced both acute lethality and more severe late radiation nephritis (fig. 3). The later the cis-Pt was given, the greater was the acute toxicity; late toxicity, however, was significantly greater when cis-Pt was given at 3 months than when it was given earlier or later (fig. 3).

A lower dose of cis-Pt (5 mg/kg) produced no acute lethality except when given 9.5 months after irradiation. At all intervals, 5 mg cis-Pt/kg decreased the latent period for fatal radiation nephritis and increased its severity. A higher dose of cis-Pt (10 mg/kg) given after irradiation produced acute lethality in all schedules and resulted in greater than 90% mortality by 12 months postirradiation. For both 5 and 10 mg cis-Pt/kg, the effects on radiation nephritis were greater when cis-Pt was given 3 months after irradiation than when it was given at other intervals.

Figure 4 shows urine creatinine levels before and after treatment of irradiated rats with 7.5 mg cis-Pt/kg. Urine

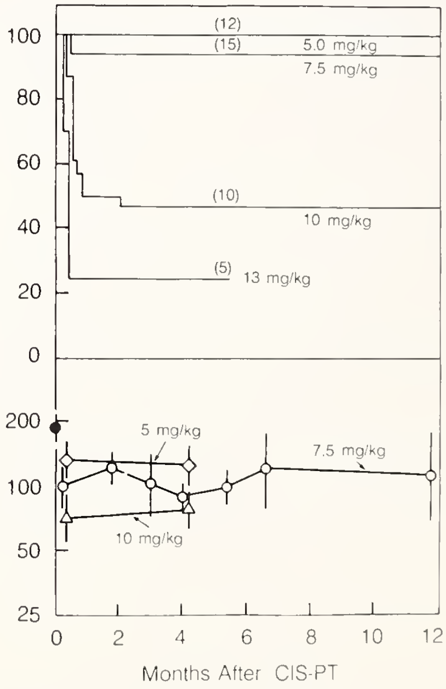


FIGURE 2.—Effect of cis-Pt on survival and renal function. *Upper panel:* Survival (percent) in control and cis-Pt treated animals. The No. of animals at risk is shown *in parentheses. Lower panel:* Urine creatinine levels (milligrams/deciliter) in control animals (●), and animals treated with 5 (◇), 7.5 (○), and 10 (△) mg cis-Pt/kg. First time point in drug-treated animals was done at 10 days. Data are shown as geometric means with 95% confidence intervals.

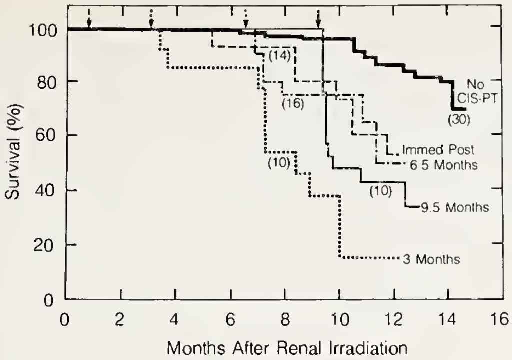


FIGURE 3.—Effect on fatal radiation nephritis of 7.5 mg cis-Pt/kg given after 9×2.22 Gy renal irradiation. The No. of animals at risk is shown *in parentheses*. Survival (percent) is shown for animals given irradiation alone (*heavy solid line*), cis-Pt 1 wk after irradiation (*dashed line*), and cis-Pt 3 mo (*dotted line*), 6.5 mo (*dash-dot line*), and 9.5 mo (*light solid line*) after irradiation.

creatinine levels immediately and 3 months after irradiation were normal; when these animals were given 7.5 mg cis-Pt/kg, urine creatinine levels decreased, but no more so than they did in unirradiated animals. At 6 and 9 months after irradiation, urine creatinine levels were decreased; treatment with cis-Pt produced a further decrease in urine creatinine, to levels less than that produced by cis-Pt alone. The acute effects on renal function of cis-Pt given after irradiation parallel the effects reported earlier for acute cis-Pt toxicity and the drug clearance after irradiation (*5*); they do not parallel the effects on chronic radiation nephritis shown in figure 3.

Both the lethality that occurred immediately after cis-Pt treatment and the lethality that occurred later were tightly correlated with abnormal renal function values (BUN, urine creatinine, urine protein). As with the irradiation alone experiments, there was no evidence for deaths due to factors other than nephritis.

Nephrotoxicity of Cisplatin Followed by Renal Irradiation

When cis-Pt was given immediately before irradiation, it had the same effect on the development of radiation nephritis as that given immediately after irradiation. Figure 5 shows that there was no statistically significant difference in survival for 7.5 mg/kg given either before or after renal irradiation. Similarly, no differences in survival were seen for 5 and 10 mg/kg given before and after irradiation. The BUN and urine creatinine levels were the same 4 months after irradiation in animals given 5, 7.5, and 10 mg cis-Pt/kg before and after renal irradiation (not shown).

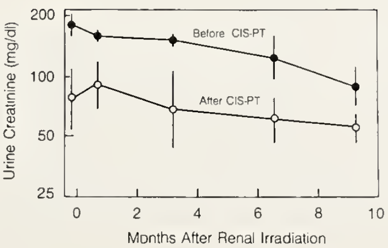


FIGURE 4.—Effect on renal function of 7.5 mg cis-Pt/kg given after 9×2.22 Gy renal irradiation. Urine creatinine levels (milligrams/deciliter) are shown before (●) and 8 days after (○) cis-Pt. Data are shown as geometric means with 95% confidence intervals.

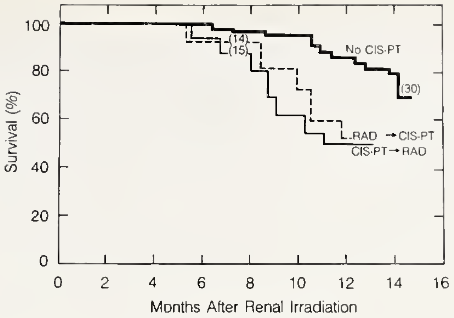


FIGURE 5.—Effect on fatal radiation nephritis of 7.5 mg cis-Pt/kg given 1 wk before (*thin solid line*) or 1 wk after (*dashed line*) 9×2.22 Gy renal irradiation. Survival of animals (percent) receiving irradiation alone is shown as a *heavy solid line*. The No. of animals at risk is shown *in parentheses*.

When the 5- and 7.5-mg cis-Pt/kg doses were given 3.7 to 7.7 months prior to fractionated renal irradiation, no effect on survival was seen. However, these animals were only followed for 9 months after irradiation, a time at which only minimal mortality is seen in animals receiving irradiation alone (fig. 1); it is possible that longer follow-up would show enhanced radiation nephritis. When a high dose of cis-Pt (10 mg/kg) was given 3.7 to 7.7 months prior to irradiation, the latent period for fatal radiation nephritis was shortened, and the severity of the nephritis was increased (fig. 6). No statistically significant difference was seen between cis-Pt administered 3.7, 5.6, or 7.7 months prior to irradiation, but none of these schedules enhanced radiation nephritis as much as cis-Pt given immediately prior to renal irradiation (fig. 6).

Four months after irradiation, urine creatinine levels were decreased in animals that received irradiation alone (fig. 7). Urine creatinine levels were also decreased 4–12 months after animals were treated with cis-Pt (fig. 7). Four

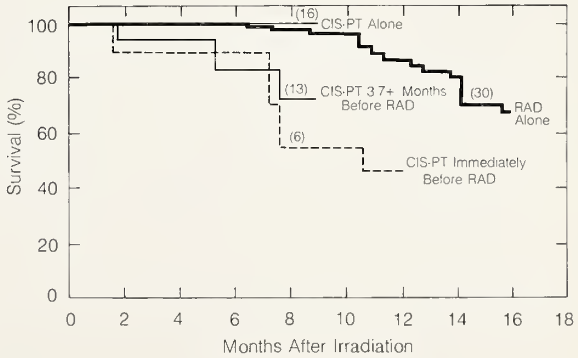


FIGURE 6.—Effect on fatal radiation nephritis of 10 mg cis-Pt/kg given 1 wk before (*dashed line*) or 3.7 to 7.7 mo before (*thin solid line*) 9×2.22 Gy renal irradiation. Animals dying from acute cis-Pt toxicity before completing irradiation are excluded from the analysis. Survival of animals (percent) receiving irradiation alone is shown as a *heavy solid line*. The No. of animals at risk is shown *in parentheses*.

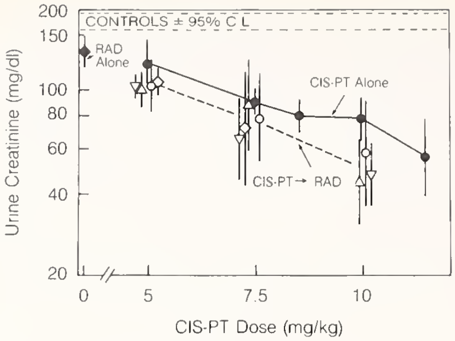


FIGURE 7.—Effect on renal function (4 mo after irradiation) of cis-Pt given prior to 9 × 2.22 Gy renal irradiation. Shown are urine creatinine levels (milligrams/deciliter) for control animals (*horizontal dashed lines*); animals receiving irradiation alone (◆); and animals receiving cis-Pt 1 wk (▽), 3.7 mo (○), 5.6 mo (△), and 7.7 mo (◇) prior to irradiation. Also shown are animals that received cis-Pt 4.5 to 11.5 mo prior to the assay (●); these animals are matched in age and time after cis-Pt treatment with the animals receiving cis-Pt followed by renal irradiation. All data are shown as geometric means with 95% confidence intervals (CL).

months after irradiation, animals that received preirradiation cis-Pt showed a greater decrease in urine creatinine levels than those that received either irradiation or cis-Pt alone (fig. 7). The effect is statistically significant in all schedules for animals given 10 mg cis-Pt/kg and for all animals that received the drug immediately before irradiation. This is consistent with the effects seen on survival (fig. 6).

DISCUSSION

We have demonstrated that cis-Pt and renal irradiation show combined toxicity even when the interval between the 2 treatments is 6 months or more. The differences between the nephrotoxicities produced by renal irradiation and cis-Pt help to explain some of the results of their combined use. The effect of cis-Pt was acute and permanent, but it was not progressive (fig. 2), whereas the effect of renal irradiation was delayed and progressive (fig. 1). Several different factors appeared to be involved when the agents were combined.

When cis-Pt was given more than 3 months prior to renal irradiation, the effect on renal function appeared to be additive. The cis-Pt treatment produced a permanent decrease in renal function (fig. 7); the progressive deficit in renal function produced by irradiation was added to this preexisting damage, and the renal function deficit reached a lethal level earlier and in a larger number of animals (fig. 6). Because cis-Pt damage was not progressive, this mechanism for combined toxicity should have produced and did produce toxicity that was independent of the interval between cis-Pt and irradiation.

When cis-Pt was given immediately before or at any time after renal irradiation, factors other than additive damage appeared to be involved. The enhanced acute toxicity of cis-Pt after renal irradiation could be explained by the previously reported effects of renal irradiation on cis-Pt clearance (*5*). Radiation effects on clearance, however, did not explain the effects of cis-Pt on the expression of radiation nephritis because the chronic effects of postirradiation cis-Pt on renal function did not parallel the change in platinum clearance. Additive toxicity would explain part of the effect; the permanent deficit in renal function from cis-Pt was added to the progressive deficit resulting from renal irradiation and gave earlier and more severe radiation nephritis. Similar additive toxicity has been reported in mice when cis-Pt was given hours to days prior to and after a single dose of renal irradiation (*4*). However, additive toxicity does not explain why cis-Pt immediately before or months after irradiation was more toxic than when the drug is given months prior to irradiation, and it does not explain the enhanced effect seen when it was given 3 months postirradiation.

These experiments do not address the issue of cis-Pt radiosensitization of the kidney (*19,20*) because cis-Pt was given 7 days before the start of fractionated irradiation and should largely have been cleared by the time irradiation began. Nor is an effect of cis-Pt on repair of radiation damage addressed by these experiments; cis-Pt was neither given during irradiation nor during the interval when radiation damage was being repaired. In this context, we wish to emphasize that sublethal radiation damage in the kidney is repaired with a half-life of about 60 minutes after doses of 1–2 Gy (*21*) and that there is no evidence of slow repair in rat (*22–24*) or mouse (*10,25*) kidney. These experiments and the work of Stewart et al. (*4*) show that renal irradiation and cis-Pt may have combined toxicity in all sequences and for all intervals; this implies that the optimal schedule for combining renal irradiation and cis-Pt should be determined by the combined effect on the tumor and suggests that great care should be taken with any such combinations.

REFERENCES

(*1*) ABELSON HT, GARNICK MB: Renal failure induced by cancer chemotherapy. *In* Cancer and the Kidney (Rieselbach RE, Garnick MB, eds). Philadelphia: Lea & Febiger, 1982, pp 769–813.

(*2*) GREENBERGER JS, WEICHSELBAUM RW, CASSADY JR: Radiation nephropathy. *In* Cancer and the Kidney (Rieselbach RE, Garnick MB, eds). Philadelphia: Lea & Febiger, 1982, pp 814–823.

(*3*) LUXTON RW, KUNKLER PB: Radiation nephritis. Acta Radiol (Ther) 2:169–178, 1964.

(*4*) STEWART F, BOHLKEN S, BARTELINK H: Renal damage in mice after treatment with cis-platinum alone or in combination with x-irradiation. Int J Radiat Oncol Biol Phys 12:927–934, 1986.

(*5*) MOULDER JE, HOLCENBERG JS, KAMEN BA, et al: Renal irradiation and the pharmacology and toxicity of methotrexate and cisplatinum. Int J Radiat Oncol Biol Phys 12:1415–1418, 1986.

(*6*) PIZZO PA, MISER JS, CASSADY RJ, et al: Solid tumors of childhood. *In* Cancer: Principles & Practice of Oncology (DeVita VT Jr, Hellman S, Rosenberg AS, eds), vol 2, 2nd ed. Philadelphia: Lippincott, 1984, pp 1511–1589.

(*7*) DOUPLE EB, RICHMOND RC: A review of interactions between platinum coordination complexes and ionizing radiation: Implications for cancer therapy. *In* Cisplatin: Current Status and New Developments (Prestayko AW, Crooks ST, Carter SK, eds). New York: Academic Press, 1980, pp 125–147.

(*8*) TEICHER BA, ROCKWELL S, LEE JB: Radiosensitization of EMT6 cells by four platinum complexes. Int J Radiat Oncol Biol Phys 11:937–941, 1985.

(*9*) DOUPLE EB, RICHMOND RC: Radiosensitization of hypoxic tumor cells by *cis*- and *trans*-dichlorodiammineplatinum(II). Int J Radiat Oncol Biol Phys 5:1369–1372, 1979.

(*10*) Williams MV, Denekamp J: Radiation induced renal damage in mice: Influence of overall treatment time. Radiother Oncol 1:355–367, 1984.

(*11*) Bulger RE, Dobyan DC: Proliferative lesions found in rat kidneys after a single dose of cisplatin. JNCI 73:1235–1242, 1984.

(*12*) Ward JM, Young DM, Fauvie KA, et al: Comparative nephrotoxicity of platinum cancer chemotherapeutic agents. Cancer Treat Rep 60:1675–1678, 1976.

(*13*) Jonas AM, Desch EL, Brennan PC, et al: Long-term holding of laboratory rodents. Inst Lab Animal Res News 29:1–26, 1976.

(*14*) Sheldon PW, Hill SA, Moulder JE: Radioprotection by pentobarbitone sodium of a murine tumor in vivo. Int J Radiat Biol 32:571–575, 1977.

(*15*) Hrushesky WJM, Levi FA, Halberg F, et al: Circadian stage dependence of *cis*-diamminedichloroplatinum lethal toxicity in rats. Cancer Res 42:945–949, 1982.

(*16*) Dance N, Proce RG, Robings D, et al: Beta-galactosidase, beta-glucosidase, and *N*-acetyl-beta-glucosamine in human kidney. Clin Chim Acta 24:189–197, 1969.

(*17*) Finney DJ: Statistical Methods in Biological Assay. New York: Hafner, 1964.

(*18*) Lee ET, Desu MM: A computer program for comparing K samples with right-censored data. Comput Methods Programs Biomed 2:315–321, 1972.

(*19*) von der Maase H: Interactions of radiation and adriamycin, bleomycin, mitomycin C or *cis*-diamminedichloroplatinum(II) in intestinal crypt cells. Br J Cancer 49:779–786, 1984.

(*20*) von der Maase H: Effect of cancer chemotherapeutic drugs on the radiation induced skin reactions in mouse feet. Br J Radiol 57:697–707, 1984.

(*21*) Moulder JE, Fish BL: Hyperfractionation and late responding normal tissues. Int J Radiat Oncol Biol Phys 10(suppl 2):121, 1984.

(*22*) Rockwell S, Moulder JE: Biological factors of importance in split-course radiotherapy. *In* Optimization of Cancer Radiotherapy (Paliwal BR, Herbert DE, Orton CG, eds). New York: Am Inst Phys, 1985, pp 171–182.

(*23*) Moulder JE: Reponse of rat kidney to fractionated radiation—lack of evidence for a time factor or for slow repair. Radiat Res 91:370, 1982.

(*24*) Moulder JE: Kidney tolerance in split-course radiation schedules. Int J Radiat Oncol Biol Phys 9(suppl 1):107–108, 1983.

(*25*) Williams MV, Stewart FA, Soranson JA, et al: The influence of overall treatment time on renal injury after multifraction irradiation. Radiother Oncol 4:87–96, 1985.

DNA Damage and Repair in Patients Receiving High-dose Cyclophosphamide and Radiation[1]

Clyde D. Ford* and C. Terry Warnick[2,3]

ABSTRACT—DNA damage and repair were assessed by alkaline sucrose gradients in the nonstimulated circulating mononuclear cells of 7 patients receiving high-dose cyclophosphamide (HDCy) and fractionated whole-body irradiation. Measurable damage produced by HDCy appeared to be repaired in about 60 hours. Damage from a radiation dose of 2 Gy was not completely repaired within 24 hours because DNA molecular weight was found to be decreased by an average of 22%. We attempted to assess the impact of HDCy on radiation damage repair by comparing blood irradiated and incubated in vitro before therapy with in vivo incubation following HDCy administration. Two hours after a radiation dose, repair appeared increased following HDCy. These results suggest the possibility that significant interaction at the DNA level may occur when HDCy and irradiation are administered together.—NCI Monogr 6:41–44, 1988.

Gamma radiation and HDCy have been used in combination to treat human and animal cancer. The interaction of these two modalities, however, is not well understood, especially at the molecular level. For example, under certain experimental conditions, HDCy has been reported to be a radiation enhancer (*1*), a radioprotector (*2*), or to have no effect (*3*). With HDCy and irradiation generally considered to produce their principal cytotoxicity by altering cellular DNA, studies of DNA damage and repair following exposure to HDCy and radiation might be useful in elucidating their individual and combined effects. In this study, we examined the induction and repair of DNA strand breaks in CMC from 7 patients receiving HDCy and fractionated whole-body irradiation prior to bone marrow transplantation.

PATIENTS AND METHODS

Patients.—Study patients consisted of 2 subjects with acute leukemia in remission, 4 with chronic or accelerated-phase, chronic myelogenous leukemia, and 1 with non-Hodgkin's lymphoma in remission. All were treated with 60 mg HDCy/kg, in the evenings of days 8 and 7 (before transplant) and 2 Gy whole-body irradiation in the mornings of days 5 to 0. After informed consent was obtained, heparinized blood was drawn from Hickman lines on the mornings indicated and immediately placed on ice. Blood was diluted 1:1 with Hanks' balanced salt solution, and mononuclear cells were separated by centrifugation over Ficoll-Hypaque. Blood was maintained at 4° C during separation procedures and until cell lysis. In some experiments, the blood was incubated at 37° C for 2 hours.

Both in vitro and in vivo irradiation were delivered at a dose rate of 7–9 rad/minute from a 10-million electron volt linear accelerator. Patient whole-body doses were calculated at the midplane with compensation for lung transmission and body geometry. Patients were treated through lateral fields while in a sitting position. Blood was uniformly irradiated in a bolus medium.

Alkaline sucrose gradients.—The alkaline sucrose gradient method, which has been described in detail elsewhere (*4*), was used with minor modification. The CMC were suspended in saline–EDTA to give approximately 10^7 cells/ml. A 5%–20% alkaline sucrose gradient containing 0.9 *M* NaCl and 0.3 *M* NaOH was prepared over a 0.5-ml cushion of 2.3 *M* sucrose. Layered on top of the gradient was 0.3 ml of an alkaline (pH 12.5) lysing solution consisting of 0.3 *M* NaCl, 0.03 *M* EDTA, 0.1 *M* Tris, 0.3 *M* NaOH, and 0.5% sarkosyl (*N*-lauroylsarcosine sodium salt; Sigma Chemical Company, St. Louis, MO). A 0.1-ml aliquot of cells was then added and covered with 0.1 ml of lysing solution. The gradient was allowed to stand in the dark for 30 minutes at room temperature, was centrifuged at 600 *g* for 2 minutes (to assure an even suspension), and then centrifuged again at 77,000 *g* for 30 minutes. To gradient aliquots of 0.4 ml were added 20 μl of 4 *N* HCl, 50 μl of bovine serum albumin (2 mg/ml), and 0.1 ml of 40% trichloroacetic acid; the resulting precipitate was allowed to form overnight. The sample was then centrifuged and the pellet washed with 5% trichloroacetic acid (twice), 95% ethanol containing 0.1 *M* sodium acetate and absolute ethanol, and then it was dried. We measured the DNA in each aliquot by letting it react with diaminobenzoic acid and then read the fluorescence produced (*5*).

From the amount of DNA in each gradient fraction, the median DNA gradient tube, i.e., the tube in the gradient where the 50th percentile of the DNA would fall, was calculated. The $S_{20,w}$ of the median gradient tube was calculated as described by McEwen (*6*). This method was found to determine the $S_{20,w}$ of λ phage to within 5% of published values. The median gradient DNA molecular weight was calculated from the median $S_{20,w}$ by the formula $S_{20,w} = 0.0528\ MW^{0.4}$ (*7*). Results were generally expressed as the

---

ABBREVIATIONS: HDCy = high-dose cyclophosphamide; CMC = circulating mononuclear cells; $S_{20,w}$ = sedimentation coefficient corrected to water at 20° C.

---

[1] Supported by the Dee Smith Foundation and Campbell Scientific.

[2] Department of Medicine, Latter-Day Saints' Hospital, Salt Lake City, UT.

[3] We thank the Latter-Day Saints' Hospital Radiation Center, Bone Marrow Transplant Unit nurses, and Sandra Sheets for their assistance.

**Reprint requests:* Clyde D. Ford, M.D., Department of Medicine, Latter-Day Saints' Hospital, Eighth Ave. and C St., Salt Lake City, UT 84143.

percent of control molecular weight. The DNA breaks/10^{12} daltons were calculated by the formula:

$$\left(\frac{\text{MW control DNA}}{\text{MW experimental DNA}}-1\right)\left(\frac{10^{12}}{\text{MW control DNA}}\right),$$

where MW = molecular weight.

Statistics.—We compared the data by using an unpaired *t*-test.

RESULTS

Relative DNA molecular weights from CMC following HDCy exposure are shown in figure 1. These were determined on alkaline sucrose gradients before and for each of 4 mornings after the first dose of HDCy. The DNA molecular weight reached a nadir on day 6 followed by progressive increase. Because patients received their first dose of irradiation on the morning of day 5, it was not possible for us to follow HDCy repair to its completion. However, the general shape of the HDCy repair curve and extrapolation of the average irradiation-induced DNA molecular weight decrease per day between days 4 and 1 back to day 5 suggested that HDCy damage was virtually completely repaired by day 4. These data suggest that maximal breaks occur at approximately 24–36 hours after a single dose of HDCy and are repaired by 48–60 hours.

On days 5 to 0, patients received total-body irradiation at a daily dose of 2 Gy. The effect of irradiation on CMC molecular weight is shown in figure 2. Because of the relatively small total amount of blood that could be obtained and the low numbers of mononuclear cells toward the end of the irradiation course, we could not study the patients each day. Patients' CMC were not able to repair completely 2 Gy damage within 24 hours. The DNA molecular weight decreased an average of 22% per day from day 4 to 1.

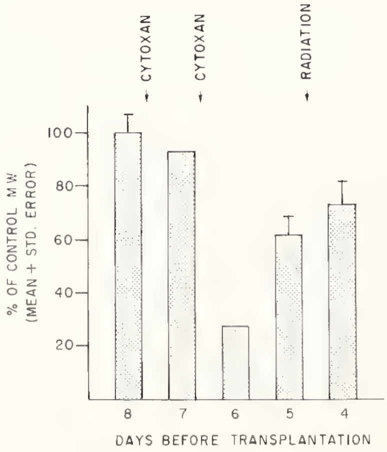


FIGURE 1.—Changes in DNA molecular weight (MW) in mononuclear cells following HDCy. *Arrows* indicate times of administration of 60 mg Cy/kg and 2 Gy total-body irradiation.

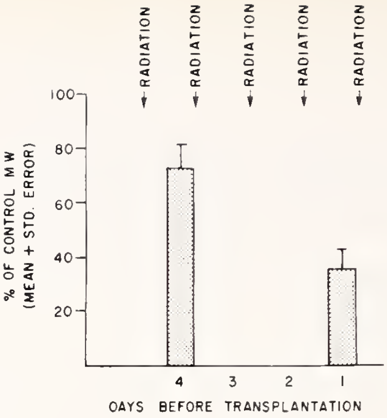


FIGURE 2.—Changes in DNA molecular weight in mononuclear cells during fractionated total-body irradiation. *Arrows* indicate times of administration of 2 Gy.

An additional goal of our study was to examine possible effects of HDCy pretreatment on irradiation repair. To establish baseline values, we had blood drawn from patients before HDCy administration; DNA molecular weight was determined before, immediately after, and 2 hours (37° C) after 2 Gy in vitro irradiation. Calculations showed that irradiation produced approximately 1 break$\cdot 10^{12}$ daltons$\cdot$rad^{-1}. These results are similar to those reported by Hashimoto et al. (*8*) for normal human lymphocytes that indicated prior chemotherapy had not significantly sensitized our patients' CMC to irradiation damage. By 2 hours, the DNA molecular weight had returned to 81% of control levels (fig. 3). Comparison with 2 normal samples, which had been cooled (4° C) for 30 minutes preirradiation, showed that a considerable amount of repair occurs even though blood at room temperature or in vivo at 37° C is placed in ice immediately following irradiation. On day 5, we had blood drawn immediately before, after, and 2 hours after the first irradiation dose to measure repair in vivo. When expressed as a percentage of the corresponding preirradiation values, DNA 2 hours after irradiation on day 5 (in vivo) was significantly higher in molecular weight ($P = .05$) than DNA 2 hours after irradiation on day 8 (in

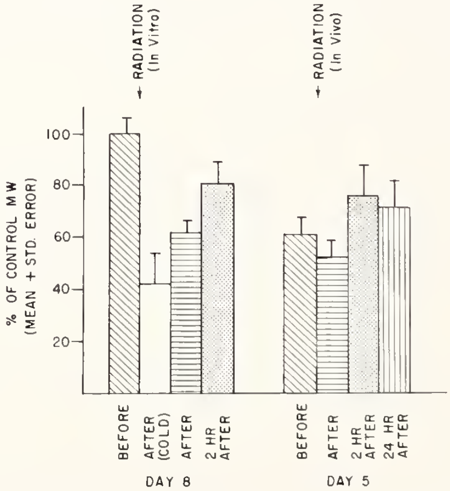


FIGURE 3.—Radiation–HDCy interaction. Day 8 represents in vitro results before HDCy was administered; day 5, in vivo results 32 hr after the last dose of HDCy. The DNA molecular weight was measured before, immediately after in cells at 4° C (cold), 2 hr after, and 24 hr after irradiation.

vitro). This suggests that CMC had an enhanced repair capability for radiation damage following HDCy. Unfortunately, we could not obtain enough CMC to determine whether this enhanced repair was still present later in the irradiation course.

DISCUSSION

The ability to repair DNA lesions is generally considered of crucial importance to the maintenance of cellular integrity in vivo. Extensive previous studies have shown that bacterial and mammalian cells have the ability to repair a variety of DNA lesions by chemical alteration or excision of damaged nucleotides. The ability of noncycling cells, such as unstimulated lymphocytes, to repair lesions induced by HDCy as judged by unscheduled DNA synthesis has been disputed (*9–11*). However, our data suggest that HDCy exposure induces breaks in mononuclear cell DNA that are subsequently repaired. The nature of these DNA breaks is not so clear. Currently, HDCy is believed to be metabolized to phosphoramide mustard and acrolein. Phosphoramide mustard produces interstrand DNA cross-links principally by alkylating the N-7 position of guanine; these cross-links are repairable (*12*). Thus we are tempted to conclude that excision repair of DNA cross-links accounts for our observed breaks and their repair. However, Erickson et al. (*12*) were unable to demonstrate the presence of DNA breaks following exposure of L1210 cells to phosphoramide mustard, which suggests that non-strand-breaking mechanisms may be operative. In addition, acrolein was reported to cause DNA breaks (*12*).

An interesting finding of our study is that detectable DNA damage from irradiation is present 24 hours after exposure. The DNA molecular weight of CMC was found to decrease by an average of 22% at 24 hours after a dose of 2 Gy. Because gamma irradiation produces free radicals that can react with sugars resulting in DNA strand breaks or with bases producing alterations susceptible to endonuclease attack (*13*), it is unclear whether strand breaks at 24 hours represent unrepairable lesions or endonuclease activity.

Although providing easily accessible tissue, human lymphocytes present potential difficulties in the interpretation of our data because of their marked heterogeneity. Previous studies have demonstrated differences in sensitivity of lymphocyte subsets to Cy (*14*), especially at low doses (*15*) and irradiation (*16*). Whether variability in DNA repair is a significant factor in lymphocyte survival following such treatments is unclear. If this is the case, at least some of the molecular weight changes we observed could be explained on this basis. Because absolute lymphocyte counts decreased by an average of 75% following Cy administration, the low DNA molecular weight observed shortly after Cy exposure may reflect cells most heavily damaged and dying, whereas surviving cells may have been more resistant to or were better able to repair DNA damage. This could have the effect of increasing molecular weight with time independent of DNA repair or could result in calculated repair kinetics which applied to the more HDCy-resistant subpopulations.

Our results suggest that the CMC that remain in vivo following HDCy have an enhanced ability to repair DNA damage. These findings are not easily interpreted, however; in vivo–in vitro differences and elimination of lymphocyte subsets with lesser repair capabilities by HDCy may be contributing factors. It is also possible that HDCy exposure is making the cells more resistant to killing by irradiation. A possible mechanism would be induction of repair enzymes similar to "adaptation" responses in bacteria (*17*) and mammalian cells (*18*). N^7-Glycosylases have been shown to be inducible in bacteria (*19*) and to excise phosphoramide mustard–guanine adducts in mammalian liver cells (*20*). Whether enhanced DNA repair is a factor in the protective effect of Cy administered prior to high-dose irradiation (*2*) or alkylating agents (*21*) will require further investigation.

There are several potential clinical implications from our study. Peters and co-workers (*22*) have shown that fractionated whole-body irradiation has its greatest therapeutic index when the fractionation interval allows time for repair of sublethal damage. If the DNA breaks remaining 24 hours after 2-Gy exposure represent continuing repair, longer irradiation intervals might be considered. Our data would also suggest that greater cell killing might occur if HDCy and radiation are given in closer proximity. Whether an increase in the therapeutic index would result in patients with cancer can only be determined in additional clinical trials.

REFERENCES

(*1*) Collis CH, Steel GG: Lung damage in mice from cyclophosphamide and thoracic irradiation: The effect of timing. Int J Radiat Oncol Biol Phys 9:685–689, 1983.

(*2*) Millar JL, Hudspith BN: Sparing effect of cyclophosphamide (NSC-26271) pretreatment on animals lethally treated with γ-irradiation. Cancer Treat Rep 60:409–414, 1976.

(*3*) Phillips TL, Ross GY, Goldstein LW: The interaction of radiation and cyclophosphamide in intestinal crypt cells. Int J Radiat Oncol Biol Phys 5:1441–1444, 1979.

(*4*) Lazarus HM, Warnick CT, Hopfenbeck A: DNA strand breakage after kidney storage. Cryobiology 19:129–136, 1982.

(*5*) Erickson LC, Osieka R, Sharkey NA, et al: Measurement of DNA damage in unlabeled mammalian cells analyzed by alkaline elution and a fluorometric DNA assay. Anal Biochem 106:169–174, 1980.

(*6*) McEwen CR: Tables for estimating sedimentation through linear concentration gradients of sucrose solution. Anal Biochem 20:114–149, 1967.

(*7*) Studier FW: Sedimentation studies of the size and shape of DNA. J Mol Biol 11:373–390, 1965.

(*8*) Hashimoto Y, Ono T, Okada S: Radiosensitivities of DNA molecules in lymphocytes from the circulating blood of man. Blood 45:503–509, 1975.

(*9*) Byfield JE, Murnane J, Ward JF, et al: Mice, men, mustards and methylated xanthines: The potential role of caffeine and related drugs in the sensitization of human tumours to alkylating agents. Br J Cancer 43:669–683, 1981.

(*10*) Ashby J, Beije B: Concomitant observations of UDS in the liver and micronuclei in the bone marrow of rats exposed to cyclophosphamide or 2-acetylaminofluorene. Mutat Res 150:383–392, 1985.

(*11*) Benigni R, Calcagnile A, Dogliotti E, et al: DNA repair induction by cytostatic drugs in proliferating and quiescent MRC-5 cells. Teratogenesis Carcinog Mutagen 3:481–490, 1983.

(*12*) Erickson LC, Ramonas LM, Zaharko DS, et al: Cytotoxicity and DNA cross-linking activity of 4-sulfidocyclophosphamides in mouse leukemia cells in vitro. Cancer Res 40:4216–4220, 1980.

(*13*) Scholes G, Chem C: Radiation effects on DNA. The Silvanus Thompson Memorial Lecture, April 1982. Br J Radiol 56:221–231, 1983.

(*14*) Kolb J-PB, Poupon M-FM, Lespinats GM, et al: Splenic modifications induced by cyclophosphamide in C3H/He, nude, and "B"-mice. J Immunol 118:1595–1599, 1977.

(*15*) Bast RC Jr, Reinherz EL, Maver C, et al: Contrasting effects of cyclophosphamide and prednisolone on the phenotype of human peripheral blood leukocytes. Clin Immunol Immunopathol 28:101–114, 1983.

(*16*) Anderson RE, Standefer JC, Tokuda S: The structural and functional assessment of cytotoxic injury of the immune system with particular reference to the effects of ionizing radiation and cyclophosphamide. Br J Cancer 53(suppl VII):140–160, 1986.

(*17*) Cairns J, Robins P, Sedgwick B, et al: The inducible repair of alkylated DNA. Prog Nucleic Acid Res Mol Biol 26:237–244, 1981.

(*18*) Frosina G, Abbondandolo A: The current evidence for an adaptive response to alkylating agents in mammalian cells, with special reference to experiments with in vitro cell cultures. Mutat Res 154:85–100, 1985.

(*19*) Karran P, Hjelmgren T, Lindahl T: Induction of a DNA glycosylase for *N*-methylated purines is part of the adaptive response to alkylating agents. Nature 296:770–773, 1982.

(*20*) Perocco P, Prodi G: DNA repair in human lymphocytes after treatment with vincristine, chlorambucil and cyclophosphamide in vitro. Haematologica 67:522–529, 1982.

(*21*) Millar JL, Hudspith BN, Blackett NM: Reduced lethality in mice receiving a combined dose of cyclophosphamide and busulphan. Br J Cancer 32:193–198, 1975.

(*22*) Peters LJ, Withers HR, Cundiff JH, et al: Radiobiological considerations in the use of total-body irradiation for bone-marrow transplantation. Radiology 131:243–247, 1979.

Long-term Consequences of Chemotherapeutic Agents on Hematopoiesis: Development of Altered Radiation Tolerance[1]

C. J. Kovacs,* M. J. Evans, J. L. Hooker, and R. M. Johnke[2,3]

ABSTRACT—The long-term effects of chemotherapeutic agents on subsequent radiation tolerance of the hematopoietic marrow were studied after a single injection of doxorubicin, 5-fluorouracil, or cyclophosphamide at a maximum tolerated dose. At designated intervals following drug treatment, drug-treated and age-matched control male B6D2F$_1$ mice were exposed to 4.5 Gy of total-body irradiation, and the recovery kinetics of the stem cell (assayed at days 8 and 13 colony-forming spleen units) and progenitor (burst-forming erythroid units, and colony-forming erythroid and granulocyte/macrophage units) compartments were established. Response deficits were calculated for each compartment by comparison of treated and control recovery curves at 5 intervals over 32 weeks. Based on these response deficits, a number of conclusions were drawn: 1) There is selective drug specificity for the more primitive (13d) and mature (8d) CFUs subpopulations; 2) these sensitivities determine the temporal consequences of drug treatment on subsequent radiation tolerance in the marrow (e.g., acute, delayed, or long term); and 3) drugs that influence long-term radiation tolerance of the marrow are dose dependent and initially affect the more primitive stem cells. The data suggest that the initial lesion in the stem cell compartment, resulting in long-term enhancement of radiosensitivity, involves a major restriction (either in cell number or in genetic functionality) of the proliferative potential necessary for recovery from subsequent radiation insult.—NCI Monogr 6:45–49, 1988.

Long-term or residual damage to a number of normal tissues resulting from treatment with specific chemotherapeutic agents has been well documented in rodents (*1–5*) and, more recently, in patients (*6,7*). These residual injuries associated with the alkylating class of drugs have been postulated to result, at least for the hematopoietic marrow, from drug-induced damage to either the stem cells (*1,5*) or hematopoietic "stromal" microenvironment (*4,8*). In either case, the drug-induced hematopoietic lesion is ultimately manifested as a proliferative defect of the dose-limiting marrow. Therefore, hematopoietic residual injuries would appear similar to those observed to occur in gastrointestinal and renal tissues following a secondary insult subsequent to drug treatment (*9,10*).

The damage to the marrow resulting in residual injury, as with other normal tissues, has been characterized as latent due to the apparent maintenance of steady state with persistent normal or near-normal peripheral blood counts and marrow cellularity. Our earlier studies (*3,11*), however, suggested that manifestation of residual injury may depend not only on the continued cell production mandated by longevity (e.g., chronic stress) but also on the magnitude of episodic, heightened proliferative activity resulting from additional acute stress such as that associated with progressive disease and its treatment. As it is likely that a majority of patients undergoing conventional treatment for malignant tumors will receive some form of chemotherapy either simultaneously or in advance of radiotherapy, it is important for the clinician to have a basic understanding of the consequences of drug damage to avoid the potential complications that may arise during and subsequent to treatment. This holds particularly true for treatment approaches when hemibody radiotherapy is used (*12,13*) and when large volumes of active marrow are exposed (*14*). For these reasons, we have examined the effects of an acute MTD of representative drugs on the subsequent response of individual marrow compartments to radiation delivered at intervals from 0 to 32 weeks after drug treatment.

MATERIALS AND METHODS

Experimental design.—Male B6D2F$_1$ (C57BL/6×DBA2) mice were given ip injections of either carrier (control) or an MTD of 10 mg ADR/kg, 250 mg CTX/kg, or 250 mg FUra/kg when the animals were 8–10 weeks old. The mice were then returned to a barrier animal housing facility and provided autoclaved laboratory chow and acid water ad libitum. At designated intervals after drug treatment over a 32-week interval, randomly selected, drug-treated, and age-matched control animals were exposed to 4.5 Gy TBI (275 kilovolt-constant potential, 15 mA; half-value layer = 0.55 copper plus 0.90 mm aluminum with a target to source distance of 51 cm). We selected the intervals between drug treatment and TBI to establish a correlation between acute (0–4 wk), delayed (4–16 wk), and residual (16–32 wk and longer) drug damage and the magnitude of hematopoietic proliferative recovery from radiation insult. Following each TBI, the size of each marrow compartment was monitored for a 28-day interval, so that we could generate individual

---

ABBREVIATIONS: MTD = maximum tolerated dose(s); ADR = doxorubicin (Adriamycin); CTX = cyclophosphamide; FUra = 5-fluorouracil; TBI = total-body irradiation; 8d = 8-day; 13d = 13-day; CFUs = colony-forming unit(s) spleen; BFUe = burst-forming unit(s) erythroid; CFUe = CFU erythroid; CFUgm = CFU granulocyte/macrophage; RD = response deficit(s); HSC = hematopoietic stem cell.

[1] Supported by Public Health Service grant CA-32903 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Division of Radiation Biology and Oncology, Radiation Oncology Center, East Carolina University School of Medicine, Greenville, NC.

[3] We thank Ms. Jean M. Joiner, Ms. Barbara M. Gould, and Dr. C. W. Scarantino for their assistance.

* *Reprint requests:* C. J. Kovacs, Ph.D., Division of Radiation Biology and Oncology, Radiation Oncology Center, East Carolina University School of Medicine, Greenville, NC 27834.

radiation response curves for the stem cell, erythroid, and myeloid progenitor compartments.

Measurement of marrow compartment size.—The methods for establishing the 8d and 13d CFUs as well as the erythroid progenitor (BFUe, CFUe) and myeloid progenitor (CFUgm) compartments of the femoral marrow have been described in detail elsewhere (*15*). Each of the marrow compartments were determined before and after a 4.5-Gy TBI dose administered to 5–10 animals, and the data are expressed as the mean compartment size/femur (SEM) for each data point.

Calculation of response deficit.—The individual data points were subjected to a Student's *t*-test for determination of the significance of difference between each set of drug-treated and control data. From the individual data, we constructed a series of radiation response curves and estimated the magnitude of the drug effect on each hematopoietic compartment by calculating an RD:

$$\mathrm{RD} = 1 - \frac{\text{(area under treated curve)}}{\text{(area under control curve)}} \times 100.$$

Under these considerations, the RD represents a proliferative defect presented as a percent; no recovery results in an RD equal to 100%.

RESULTS

5-Fluorouracil and Radiation Tolerance: Acute Drug Damage

The major influence of an MTD of FUra on subsequent radiation tolerance of the marrow was observed during the first 1- to 2-week interval following drug treatment (fig. 1). Those RD of 90% or more were estimated for all compartments of the marrow with the exception of the 13d CFUs subpopulation for which the RD was about 25%. During the succeeding 2–3 weeks, RD were reduced in all compartments to 50% or less and, with the exception of the CFUgm, continued to decline through 32 weeks, when the response to 4.5 Gy was indistinguishable from that of age-matched control marrow. The RD of the CFUgm compartment, however, increased between 4 and 8 weeks after drug treatment and remained elevated throughout the remaining 24-week interval, which suggests that FUra exerts a major and apparently sustained effect on the proliferative recovery potential of the WBC progenitor in the marrow.

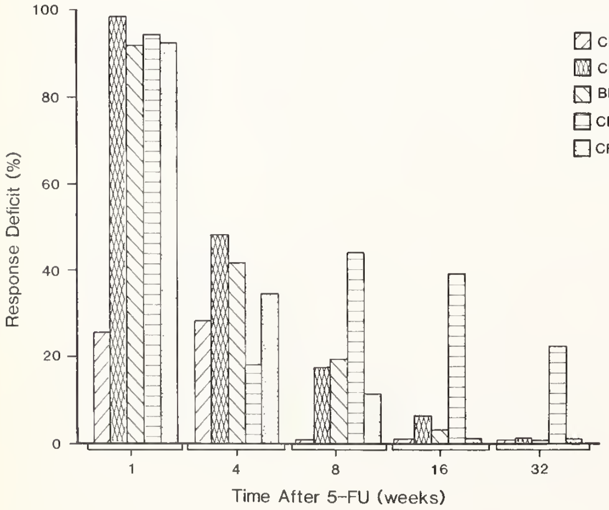


FIGURE 1.—Effect of a single MTD of 250 mg FUra/kg on the subsequent radiation RD of the hematopoietic compartments. Significance of difference between the treated and control data at each time point (0–28 days) used to generate the response curves was established by a Student's *t*-test. Each control and treated time point following 4.5 Gy of TBI was obtained from analysis of the marrow of 5–10 animals. Irradiation occurred at either 1, 4, 8, 16, or 32 wk after drug treatment.

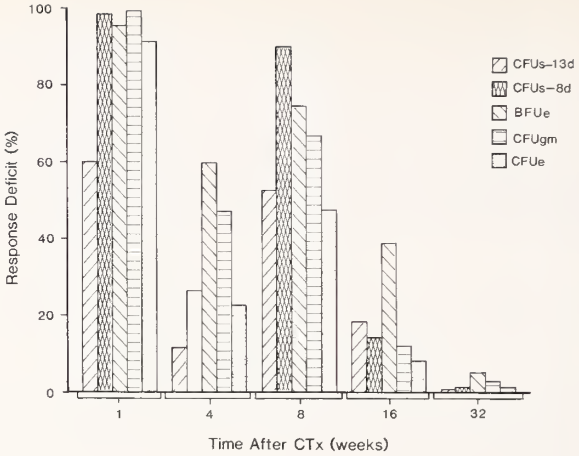


FIGURE 2.—Effect of a single MTD of 250 mg CTX/kg on the subsequent radiation RD of the hematopoietic compartments. Experimental conditions are identical to those described in fig. 1.

Cyclophosphamide and Radiation Tolerance: Delayed Drug Damage

Similar to FUra, acute damage resulting from an MTD of CTX also significantly influenced the radiation tolerance of the marrow during the first 1–2 weeks following drug treatment. With the exception of the 13d CFUs subpopulation, RD of 90% or more occurred in all compartments. The CTX-induced RD of the 13d CFUs, though significantly less than that estimated for the other compartments, was higher (60% vs. 25%) than the RD resulting from FUra (fig. 2). During the subsequent 2–3 weeks, the RD decreased in all compartments to 60% or less, with the RD of the 8d and 13d CFUs subpopulations considerably more reduced than that for either the BFUe or CFUgm progenitor compartment. Unlike the observations made following FUra, however, the RD for all marrow compartments increased again at 8 weeks after CTX treatment. The most significant increases occurred in the 8d (90% vs. 27%) and 13d (52% vs. 12%) CFUs subpopulations. These increases at 8 weeks were followed by a progressive decrease in RD for all compartments until, at 32 weeks, the influence of CTX on the radiation tolerance of the marrow was insignificant.

Doxorubicin and Radiation Tolerance: Residual Drug Damage

At the MTD, ADR also demonstrated a biphasic influence on the radiation tolerance of marrow (fig. 3). However, major differences in the magnitude and temporal appearance of RD in the individual compartments of the

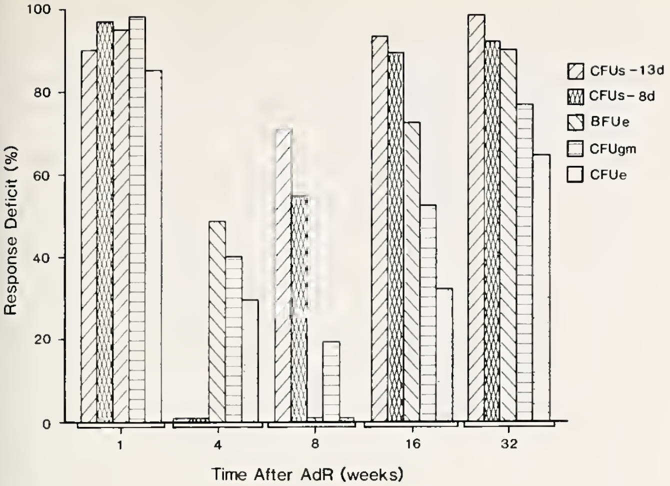


FIGURE 3.—Effect of a single MTD of 10 mg ADR/kg on the subsequent radiation RD of the hematopoietic compartments. Experimental conditions are identical to those described in fig. 1.

marrow were observed following treatment with this drug. One major difference occurred during the acute phase (0–4 wk) of drug effect. When compared with either of the other 2 drugs used, ADR induced a marked increase in the RD of the 13d CFUs (RD=25%, 60%, and 91% for FUra, CTX, and ADR, respectively). The second difference was observed at 4 weeks after ADR when, unlike CTX, the RD for both the 8d and 13d CFUs were essentially indistinguishable from those of age-matched control marrow. Collectively, these observations suggested that the acute effects of ADR on the CFUs compartment (but not the progenitor compartments) were more rapidly eliminated than those resulting from either FUra or CTX.

Elevated RD reappeared at 8 weeks after ADR treatment in both the 8d and 13d CFUs, as well as in the CFUgm. The RD in the 13d CFUs at this time, unlike that observed at comparable times after CTX, was greater than the RD in the 8d CFUs (72% for the 13d vs. 54% for the 8d CFUs). By 16 weeks after ADR, elevated RD were present in all the marrow compartments. Over the subsequent 16-week period, RD continued to increase until at 32 weeks after drug treatment the RD for all compartments were, with the exception of the CFUgm and CFUe, 90% or more. Arrangement of the individual compartments in decreasing order of their RD magnitude (13d CFUs > 8d CFUs > BFUe > CFUgm > CFUe) provided an index of the long-term effects of ADR on the marrow compartments.

DISCUSSION

The data generated from these studies of representative chemotherapeutic agents have demonstrated that each drug produces a temporally unique effect on the hematopoietic marrow that is manifested by corresponding alterations in hematopoietic radiation tolerance. In each case, the selective specificity of the drug and the subsequent effects on radiation tolerance can be explained by the age-generation hypothesis (*16*) for the hematopoietic stem cell compartment where subpopulations of stem cells are arranged in a concatenated continuum from the most primitive to the most mature cells on the basis of self-renewal potential.

Of the 3 drugs studied, only FUra could be considered to produce what has been conventionally referred to as acute damage (fig. 1). The long-term effects of this drug on radiation tolerance are in agreement with the more immediate sensitivity of the 8d (vs. the 13d) CFUs and CFUgm to FUra (*17–19*). In fact, this preferential sensitivity of the more mature CFUs and committed progenitor of the granulocyte/macrophage has been a major resource in establishing the presently accepted model of the HSC hierarchy (*16,19*). From observations made with FUra, the limits of the HSC compartment are postulated to extend from a true totipotential subpopulation with extensive proliferative (self-renewing) potential to a monopotential but multilineage 8d CFUs with negligible self-renewal potential. The 13d CFUs, estimated during these studies, exists somewhere to the left of the 8d CFUs in the HSC continuum as a more primitive subpopulation in this hierarchy, and is responsible, via differentiation, for replenishing blood cells under emergency as well as day-to-day conditions (*16*). Based on this model, it is conceivable that FUra would continue to result in RD in those compartments preferentially sensitive to the drug until recovery from the acute effects of the drug had been completed. The failure of the RD in the 13d CFUs to decrease concomitantly with a marked reduction of the RD of all other compartments suggests that the 13d CFUs, as reported earlier (*16*), actively participate in the recovery of the 8d CFUs, BFUe, and CFUgm for at least 4 weeks. Thereafter, recovery of the CFUgm appears dependent on the 8d CFUs.

Unlike FUra, an MTD of CTX manifested both acute damage as well as a subsequent transient damage to the hematopoietic marrow (fig. 2). The first of these biphasic influences exerted on radiation tolerance by CTX, similar to that observed following treatment with FUra, could be ascribed to the acute effects of the drug on the individual marrow compartments. Whereas CTX and FUra have similar effects on the initial survival (*20*) and RD (fig. 1, 2) of the 8d CFUs, the RD of the 13d CFUs at 1 week after CTX was significantly greater than that observed following FUra

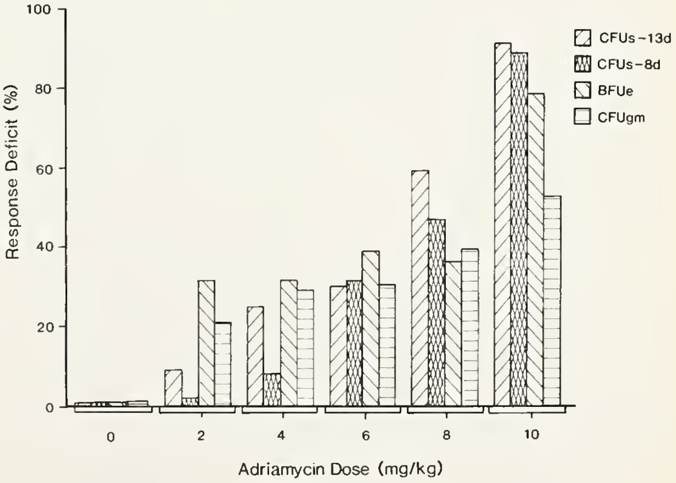


FIGURE 4.—Effect of the dose of ADR on the radiation RD of the hematopoietic compartments at 16 wk after drug treatment. Experimental conditions are identical to those described in fig. 1. Irradiation occurred at 16 wk after treatment with ADR.

treatment. The differences observed between these 2 drugs would suggest that the 13d CFUs is more sensitive to CTX than to FUra and would explain, at least partly, why repopulation of the 8d CFUs occurs more rapidly following FUra than after treatment with CTX. Furthermore, it could also explain why the self-renewal capacity of the marrow is more affected by CTX than by FUra (*20*). Unfortunately, evaluation of the temporal response of the acute effects of CTX on the 13d CFUs beyond 4 weeks was complicated by the reappearance of an elevated RD at 8 weeks after treatment in this subpopulation as well as in the other marrow compartments. The second influence of CTX noted on the radiation tolerance of the marrow (8–16 wk) resulted in RD in the 8d and 13d CFUs similar in magnitude to those occurring during the acute phase (1–2 wk). By comparison, RD in the committed progenitor compartments were, at the same time, less than those observed during the acute phase of toxicity.

Although these observations suggested that the appearance of this delayed effect of CTX resulted from drug effects on the HSC compartment, CTX is also reported to produce a lesion in the hematopoietic stromal function of the marrow that could influence the radiation response of the marrow. These stromal defects, unlike the damage to the 8d CFUs, are not recovered by 6 weeks after drug treatment (*8*). Furthermore, in studies similar to those reported here, CTX was observed to result in a small but significant decrease in the proliferative potential of the 8d CFUs (*4*). Due to the limited self-renewal of the 8d CFUs, however, it is conceivable that the primary effects of CTX, as demonstrated here (fig. 2), were to the more primitive CFUs, which were subsequently reflected in the 8d CFUs subpopulation. In either case, the earlier (*4,8*) and the preliminary studies (unpublished data) from this laboratory have demonstrated a transient but progressive lesion in the stromal compartment of the marrow following treatment with CTX. The temporal similarities between the second wave of RD in the marrow and the duration of the stromal defect collectively support the hypothesis that the delayed effects of CTX on the marrow compartments observed as RD in these studies may be the result of transient damage to the microenvironmental stroma.

The development of radiation RD in the marrow during the 32-week interval following treatment with an MTD of ADR was consistent with the temporal changes observed in the CFUs compartment (fig. 3). Following such a dose, two periods of CFUs depression are evident in the marrow; the first occurs immediately after drug treatment with recovery within 3–4 weeks, the second commences at 16 weeks after treatment, but, unlike the first, gradually progresses until at 32 weeks RD of approximately 50% of age-matched control marrow exist (*21,22*). Although the initial depression of the 8d and 13d CFUs can again be explained by the acute effect of the drug on the marrow, the delayed appearance of the second CFUs depression, following an interim of 12 weeks at normal levels, suggests that either 1) a second drug-induced lesion has developed in the marrow with time, or 2) full manifestation of the initial lesion is transiently masked. The progressive increase in RD of both CFUs subpopulations following 4.5 Gy at a time when the marrow content of 8d and 13d CFUs is normal, however, suggests that even though the lesion may already exist, albeit at what the RD predicts is a reduced level, it requires a significant proliferative stimulus for manifestation. Furthermore, based on a hierarchy of stem cells, it would appear that the major lesion resulting in RD in the marrow does not reside in either the 8d or 13d CFUs subpopulations.

At least two explanations are appropriate for these observations, both recognizing the HSC compartment as a true continuum ranging from the very young (high self-renewal) to the oldest (negligible self-renewal) stem cells. The first would suggest that ADR, initially affecting all HSC subpopulations, preferentially reduces the proliferative potential of the HSC by restricting (either by elimination or genetic alteration) the most primitive subpopulation of the HSC hierarchy or at least those cells more primitive than the 13d CFUs. Due to the self-renewing capacity of the surviving HSC subpopulations, however, hematopoietic integrity would continue until the proliferative potential of the marrow was sufficiently exhausted by normal or insult-related proliferation. With ADR, this would also explain the maintenance of the CFUs through 16 weeks prior to their progressive decline, as well as the progressive increase in RD to 4.5 Gy as the delivery of radiation insult approached 32 weeks. This explanation of the data is further supported by the observation that RD for both CFUs subpopulations, but not the progenitor compartments, were dose dependent, and that the RD of the 13d CFUs resulted from lower doses of ADR than those required to produce RD in the 8d CFUs subpopulation (fig. 4).

The second explanation of the observations reported here would involve a progressive lesion in the stromal microenvironment of the marrow. Evidence for long-term damage to the stromal elements of the marrow following chemotherapy and radiotherapy have been previously observed by investigators (*23–26*) using long-term bone marrow cultures. The stromal defects observed in these cultures prepared from marrow treated with busulfan (*25*) and ADR (Evans MJ, unpublished data) significantly influence the production of total as well as granulocyte progenitor cellularity in the long-term bone marrow cultures. Recharging these cultures with age-matched (either treated or control) marrow results in either an abatement (control marrow) or enhancement (treated marrow) of the stromal lesion (*25*); however, unequivocal experimental definition of the residual stromal lesion in them has not been established. This may be due in part to an apparent "stromal-generating" potential of the recharging, age-matched, control marrow (*25*). Preliminary data from this laboratory suggest that the functional integrity resulting from recharging (with normal marrow) those stromal cultures established sequentially from femurs over a 32-week interval following ADR treatment is progressively compromised. Furthermore, in vivo evidence for the existence of a prolonged (but transient) defect in the microenvironment of the marrow following CTX has been provided (*4,8*). Collectively, therefore, these observations strongly support the hypothesis that a stromal lesion, as well as one in the HSC, may occur following treatment with specific chemotherapeutic agents. Drug-induced, stromal lesions in other normal tissues may also explain the temporal similarities between the development of residual drug damage in the renal and gastrointestinal epithelia (*9,10*) and that reported here for the hematopoietic marrow.

CONCLUSIONS

Using radiation therapy as a proliferative stress and potential therapeutic modality, we determined the temporal consequences of chemotherapy experimentally, either alone or in combination with radiation, for 3 representative and clinically relevant drugs at an MTD. Collectively, the data supported the widely held concept that drug toxicity exists in three modes, i.e., acute, delayed, and residual. Furthermore, it was apparent that some form of acute toxicity was associated with each agent but that the sensitivity of individual compartments of the marrow differed for each drug. More importantly, the data demonstrated that the initial drug sensitivity of the individual CFUs subpopulations of the HSC hierarchy predicted the radiation tolerance of the marrow subsequent to drug treatment and that these altered periods of radiosensitivity corresponded to the three temporal sequelae of drug toxicity. Drugs affecting the more primitive (13d CFUs) HSC subpopulations (e.g., ADR) resulted in an enhanced radiosensitivity of the marrow both immediately (*acute;* 0–4 wk) and progressively over prolonged (and perhaps permanent) intervals (*residual;* 16–32 wk or longer), but those drugs selectively sensitive to the mature HSC (e.g., FUra, CTX) only resulted in a close-order (*acute*) enhancement of marrow radiosensitivity. Drugs producing a transient, but prolonged, stromal effect (e.g., CTX), in addition to reducing the size of the more mature HSC subpopulations, resulted in a second, but transient, interval of enhanced radiosensitivity, which, due to its temporal positioning between the *acute* and *residual* effects, was considered to represent a delayed (8–16 wk) effect. However, the contribution of the stromal defect on residual damage remains unclear.

REFERENCES

(*1*) Botnick LE, Hannon EC, Hellman S: Limited proliferation of stem cells surviving alkylating agents. Nature 262:68–69, 1976.

(*2*) Trainor KJ, Morely AA: Screening of cytotoxic drugs for residual bone marrow damage. J Natl Cancer Inst 57:1237–1239, 1976.

(*3*) Kovacs CJ, Schenken LL, Evans MJ, et al: Enhanced Adriamycin-induced delayed gastrointestinal radiosensitivity in tumor-bearing animals. Int J Radiat Oncol Biol Phys 7:1389–1395, 1981.

(*4*) Fried W, Barone J: Residual marrow damage following therapy with cyclophosphamide. Exp Hematol 8:610–614, 1980.

(*5*) Botnick LE, Hannon EC, Hellman S: Nature of the hematopoietic stem cell compartment and its proliferative potential. Blood Cells 5:195–210, 1979.

(*6*) Slavin RE, Dias MA, Saral R: Cytosine arabinoside induced gastrointestinal toxic alterations in sequential chemotherapeutic protocols. Cancer 42:1747–1759, 1978.

(*7*) Hayworth C, Morris-Jones PH, Testa WG: Long-term bone marrow damage in children treated for ALL: Evidence from in vitro colony assays (GM-CFC and CFU-F). Br J Cancer 46:918–923, 1982.

(*8*) Fried W, Kedo A, Barone J: Effects of cyclophosphamide and of busulfan on spleen colony-forming units and on hematopoietic stroma. Cancer Res 37:1205–1209, 1977.

(*9*) Schenken LL, Burhold DB, Kovacs CJ: Adriamycin-radiation combinations: Drug-induced delayed gastrointestinal sensitivity. Int J Radiat Oncol Biol Phys 5:1265–1269, 1979.

(*10*) Kovacs CJ, Braunschweiger PG, Schenken LL, et al: Proliferative defects in renal and intestinal epithelium after *cis*-dichlorodiammine platinum(II). Br J Cancer 45:286–294, 1982.

(*11*) Kovacs CJ, Johnke RM, Evans MJ, et al: Development of latent residual drug damage to the hematopoietic marrow during the subsequent growth of tumors. Exp Hematol 14:165–172, 1986.

(*12*) Salazar O, Creech R, Rubin P, et al: Half-body and local chest irradiation as consolidation following response to standard induction chemotherapy for disseminated small cell lung cancer. An Eastern Cooperative Oncology Group pilot report. Int J Radiat Oncol Biol Phys 6:1093–1102, 1980.

(*13*) Salazar O, Rubin P, Keller B, et al: Systemic (half-body) radiation therapy: Response and toxicity. Int J Radiat Oncol Biol Phys 4:950–973, 1978.

(*14*) Woodard HQ: The relation of weight of hematopoietic marrow to body weight. Br J Radiol 57:903–907, 1984.

(*15*) Kovacs CJ, Johnke RM, Emma DA, et al: Residual Adriamycin (AdR)-induced hematopoietic damage: A consideration for subsequent radiotherapy. Int J Radiat Oncol Biol Phys 11:1955–1961, 1985.

(*16*) Rosendaal M, Hodgson GS, Bradley TR: Organization of haemopoietic stem cells: The age-generation hypothesis. Cell Tissue Kinet 12:17–29, 1979.

(*17*) Hodgson GS, Bradley TR: Properties of haematopoietic stem cells surviving 5-fluorouracil treatment: Evidence for a pre-CFU-S cell? Nature 281:381–382, 1979.

(*18*) Van Zant G: Studies of hematopoietic stem cells spared by 5-fluorouracil. J Exp Med 159:679–690, 1984.

(*19*) Hodgson GS, Bradley TR: The in vivo kinetic status of hematopoietic stem and progenitor cells as inferred from labelling with bromodeoxyuridine. Exp Hematol 12:683–687, 1984.

(*20*) Botnick LE, Hannon EC, Vigneulle R, et al: Differential effects of cytotoxic agents on hematopoietic progenitors. Cancer Res 41:2338–2342, 1981.

(*21*) Kovacs CJ, Johnke RM, Emma DA, et al: Effect of prolonged expansion of marrow progenitor compartments following doxorubicin (AdR) on subsequent radiation tolerance. Exp Hematol 13:89–94, 1985.

(*22*) Kovacs CJ, Evans MJ, Hooker JL, et al: A permanent defect in T-cell function resulting from Adriamycin (AdR). Exp Hematol 14:504, 1986.

(*23*) Mauch P, Greenberger JS, Botnick L, et al: Evidence for structural variation in self-renewal capacity within long-term marrow cultures. Proc Natl Acad Sci USA 77:2927–2930, 1980.

(*24*) Reincke U, Hannon EC, Hellman S: Residual radiation injury exhibited in long-term bone marrow cultures. J Cell Physiol 112:345–352, 1982.

(*25*) Hays EF, Hale L, Villarreal B, et al: "Stromal" and hematopoietic stem cell abnormalities in long-term cultures of marrow from busulfan-treated mice. Exp Hematol 10:383–392, 1982.

(*26*) Testa NG, Hendry JH, Molineux G: Long-term bone marrow damage in experimental systems and in patients after radiation or chemotherapy. Anticancer Res 5:101–110, 1985.

Modification of Cyclophosphamide-induced Pulmonary Toxicity in Normal Mice[1]

M. Joan Allalunis-Turner* and Dietmar W. Siemann[2,3]

ABSTRACT—**The effects of fractionated doses, in vivo thiol modulation, and antifibrinolytic therapy on the expression of lung damage induced by cyclophosphamide (Cy) were evaluated in C3H mice. The protein content of lung lavage samples taken 4 days after Cy treatment was used as an early indicator of damage. In fractionation studies, little difference in lung protein was observed when 200 mg of Cy/kg was administered as a single dose or as two or four equal doses given daily, suggesting that little sparing effect occurred with fractionated doses of Cy. In experiments that tested the effects of exogenous thiol administration, mice treated with WR-2721 before Cy were protected against lung damage, whereas the use of sodium thiosulfate or mesna did not give this protection. Treatment with ϵ-aminocaproic acid, which inhibits the breakdown of fibrin clots, did not result in enhanced Cy damage as measured by lung lavage or breathing rate; this suggests that the extended presence of fibrin per se did not contribute to Cy-induced pulmonary damage.—NCI Monogr 6:51-53, 1988.**

A significant increase in pulmonary toxicity has been observed following aggressive combined modality treatments such as those used for small cell lung cancer and in the conditioning of patients for bone marrow transplantation (*1–3*). Both radiation and chemotherapeutic agents are recognized as contributing to lung damage, although the identity of the target cells and the contribution of each treatment to the final expression of damage remain uncertain. Most previous work has focused on the enhancing effects of chemotherapy on radiation-induced lung damage. Relatively less attention has been paid to the effects of chemotherapeutic agents alone. Because Cy is an agent that is common to many combined modality protocols, we have investigated the effects of Cy on normal mouse lung. Specifically, experiments were designed to determine if Cy-induced lung damage could be modified by fractionating the dose of Cy, by pretreating with exogenous thiol compounds, or by maintaining Cy-treated animals on an antifibrinolytic agent. Previous studies from this laboratory have established that a dose-dependent increase in total lung protein follows treatment with Cy. This increase, which peaks at day 4, is positively correlated with both increases in breathing rate and the occurrence of pulmonary pneumonitis and fibrosis (*4*). In these studies, the correlation between increased breathing rate and lung protein was also observed. Therefore, changes in total lung lavage protein content were used as an early indicator of lung damage.

MATERIALS AND METHODS

Animals and Drug Treatments

For these experiments, 8- to 12-week-old female C3H/HeJ mice obtained from Jackson Laboratories were used. All drugs were dissolved in 0.9% NaCl immediately before use and were administered ip on the basis of animal body weight. Control animals received equivalent volumes of NaCl. In studies of the effects of fractionated Cy doses on lung damage, mice received one of three treatments with Cy: a single dose of 200 mg/kg on day 0; 100 mg/kg on days 0 and 2; or 50 mg/kg on days 0–3. In studies of the effects of thiol compounds on lung damage induced by Cy (300 mg/kg), mice received WR-2721 (National Cancer Institute), STS (Sigma Chemical Co.), or mesna (Sigma Chemical Co.), according to the following schedule: WR-2721 (400 mg/kg) 30 minutes before Cy; STS (1,600 mg/kg) 10 minutes before Cy; or mesna (900 mg/kg) 30 minutes before Cy. To evaluate the in vivo distribution of STS, animals were injected with ^{35}S-labeled STS (Amersham Corp.; specific activity, 1.05×10^{-4} Ci/mg) at a dose of 53 μCi per mouse. Lungs were removed 5–60 minutes after injection, and STS uptake by the lung was determined using a scintillation counter (Beckman Instruments, Inc.). In separate studies, EACA (Sigma Chemical Co.) was used to assess the effects of combining an antifibrinolytic agent with Cy treatment. In assays that used increases in lung lavage protein as an end point, EACA (500 mg/kg) was injected 30 minutes before Cy (300 mg/kg) or 30 minutes before Cy and daily until the time of assay. In experiments that assessed the effect of EACA on Cy-induced alterations in breathing rate, EACA (500 mg/kg) was injected 30 minutes before graded doses of Cy (250–300 mg/kg) and daily thereafter for 2 weeks.

Breathing Rate Measurements

The breathing frequency of animals was determined using a plethysmograph as previously described (*5*). Measurements were begun 4 days after the first dose of Cy and were continued at weekly intervals.

ABBREVIATIONS: Cy = cyclophosphamide; STS = sodium thiosulfate; EACA = ϵ-aminocaproic acid; GSH = glutathione.

[1] Supported by Public Health Service grant CA-38637 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services. M. J. Allalunis-Turner is a recipient of an Alberta Foundation for Medical Research Fellowship.

[2] Experimental Therapeutics Division, Department of Radiation Oncology, University of Rochester Cancer Center, NY.

[3] We thank S. Berman, S. Curnick, and A. Flaherty for technical assistance.

* *Reprint requests:* M. Joan Allalunis-Turner, Ph.D., Cross Cancer Institute, 11560 University Ave., Edmonton, Alberta T6G 1Z2, Canada.

Lung Lavage Protein Assay

Mouse lungs were perfused free of blood by injecting NaCl into the heart. Excised lungs were then lavaged with 2.1 ml of ice-cold NaCl. Following centrifugation, the cell-free lavage material was assayed for protein content as previously described (*6*).

RESULTS

Mice were injected with Cy (200 mg/kg) given as a single dose or as fractionated doses, and the amount of lung lavage protein was determined 4 days after the initiation of the treatment. Table 1 shows that a single dose of Cy resulted in a significant increase ($P<.05$) in total lung lavage protein, compared to control values. When the total dose of Cy was fractionated into two or four smaller doses, little sparing effect was evident because the amount of protein in the lavage material from animals that received fractionated doses of Cy was not significantly different from the amount of protein recovered from animals after a single dose of Cy.

In experiments designed to determine if selected thiol compounds could protect against lung damage, mice were injected with WR-2721, STS, or mesna before Cy (300 mg/kg), and lung lavage protein was determined 4 days later. Table 1 shows a significant increase ($P<.005$) in total alveolar protein from Cy-treated animals, compared to that obtained from controls. Pretreatment with WR-2721 (400 mg/kg) reduced the total amount of protein recovered in lavage samples to a value not significantly different from that of the controls, while pretreatment with STS (1,600 mg/kg) or mesna (900 mg/kg) had no protective effect.

The effect of EACA on Cy lung damage also was evaluated. In the group that received a single dose of EACA before Cy, no significant change in lavage protein content was observed, compared to the group treated with Cy only (table 1). When EACA was injected both before Cy and daily thereafter, a modest reduction in lavage protein was observed. However, the decrease in total protein that followed multiple EACA treatments was not significant and could not be correlated with a protective effect when later functional changes were assessed. Specifically, the breathing rate of animals maintained on daily doses of EACA following treatment with a range of Cy doses was not significantly different from that of animals that received Cy only (data not shown).

TABLE 1.—Effects of fractionated doses, in vivo thiol modulation, or antifibrinolytic therapy on Cy-induced pulmonary damage

Treatment	Total lung lavage protein (mg)[a]
Fractionated Cy	
Control	0.66 ± 0.17
Cy (200 mg/kg × 1)	1.20 ± 0.09
Cy (100 mg/kg × 2)	0.98 ± 0.13
Cy (50 mg/kg × 4)	0.97 ± 0.16
Exogenous thiol compounds	
Control	0.97 ± 0.11
Cy (300 mg/kg)	2.08 ± 0.49
Cy (300 mg/kg) + WR-2721 (400 mg/kg)	0.95 ± 0.19
Cy (300 mg/kg) + STS (1,600 mg/kg)	1.54 ± 0.23
Cy (300 mg/kg) + mesna (900 mg/kg)	2.43 ± 0.34
EACA treatment	
Control	0.53 ± 0.12
Cy (300 mg/kg)	2.16 ± 0.96
Cy (300 mg/kg) + EACA (500 mg/kg × 1)	1.74 ± 0.64
Cy (300 mg/kg) + EACA (500 mg/kg × 5)	1.18 ± 0.11

[a] Values = $\bar{x} \pm$ SEM.

DISCUSSION

These studies have sought to determine whether Cy-induced lung damage could be modified by fractionated drug treatments, the use of exogenous thiols, or antifibrinolytic therapy. In the first approach, a fixed total of Cy was administered as a single dose or as multiple doses. This scheduling was patterned after that used in the conditioning of patients for bone marrow transplantation; in this scheduling, large single or small multiple doses of Cy are used to ablate the hematopoietic system. All treatment protocols resulted in pulmonary toxicity, suggesting that while damage may decrease with fractionation, the effect is small and therefore may not effectively abrogate Cy-induced lung damage. Our studies also suggest that little recovery of Cy damage occurred between treatment intervals. Collis and co-workers (*7*) have previously shown that pretreatment with a small conditioning dose of Cy can protect mouse lungs against subsequent challenge with larger doses. Based on these studies, they have proposed a model of lung repair kinetics suggesting that some repair of Cy damage by the alveolar epithelium may occur within 24 hours. In our studies, which used lavage protein as an early indicator of lung damage, no significant difference was observed in the level of damage after a large single dose or multiple doses administered 24 or 48 hours apart. The doses used in our treatment schedule were somewhat larger than those used by Collis et al., and this may explain our inability to observe a protective effect with fractionation. However, present results are consistent with the clinical observation that the total accumulated dose of Cy dictates the extent of lung damage (*8*).

The failure of dose-fractionated Cy treatment to significantly protect the lung led to studies of the potential protective effects of the thiol compounds WR-2721, STS, and mesna. Use of WR-2721 before irradiation has been shown to confer significant protection against the late manifestations of lung damage (*9*). In the present studies with WR-2721, significant protection was achieved when animals were treated with WR-2721 before Cy, as lung lavage protein levels obtained in these animals were reduced to near control values. This was consistent with breathing rate measurements from Cy-treated animals. In the absence of WR-2721, breathing rates of mice treated with Cy (300 mg/kg) were 1.4 times above control values. In contrast, the breathing rates of mice treated with WR-2721 before Cy were not different from those of controls. However, the results of preliminary experiments designed to examine the in vivo interactions of WR-2721 and Cy suggest that a component of this protection may be attributable to an alteration by WR-2721 of Cy pharmacokinetics (Allalunis-Turner MJ, Siemann DW, unpublished observations). Therefore, while the observed protection of the lung by WR-2721 is encouraging, the possibility that an alteration in drug pharmacokinetics may result in a reduction of tumor cell kill needs to be considered.

In this study, STS and mesna were investigated as potential protectors against pulmonary toxicity because other workers had demonstrated that protection of normal bladder tissue against Cy damage could be achieved if these agents were administered before Cy (*10–12*). With these drugs, protection is thought to result from the replenishing of thiol stores, which are depleted by acrolein, a metabolite of Cy. If acrolein is the Cy metabolite responsible for damage to normal lung, as is the case for normal bladder, then treatment with STS and mesna might be expected to result in protection. Studies from this laboratory have demonstrated that following treatment with a single dose of Cy (300 mg/kg), lung GSH is reduced to approximately 60% of control values. Berggren and colleagues (*13*) have shown that isolated rat lungs depleted of GSH by diethylmaleate can utilize perfused exogenous thiol compounds to replenish intracellular GSH stores. Consequently, if depletion of GSH by Cy contributes to lung damage, then STS and mesna might afford some lung protection. However, STS and mesna failed to protect against Cy. There was concern that the binding of STS and mesna to blood plasma proteins may have prevented delivery of effective concentrations to the lung. Whether appreciable quantities of mesna reached the lung remains to be determined. However, our results with labeled STS indicate that a significant quantity of the drug was present in the lung at the time of Cy treatment. The 10-minute time interval between STS and Cy was chosen because this time corresponds to peak STS concentration in the lung. In spite of this, no lung protection was observed. This finding has suggested that acrolein, the metabolite thought to be responsible for bladder toxicity, does not contribute in a significant way to lung damage. In preliminary experiments designed to test this hypothesis, we have noted that deschloro-4-hydroperoxy-cyclophosphamide, a compound with metabolism that results in the production of acrolein but not an active alkylating species, failed to induce pulmonary toxicity (Siemann DW, Allalunis-Turner MJ, in preparation). More extensive studies are in progress to characterize the role of specific Cy metabolites in the generation of lung damage. However, this observation coupled with the lack of protection by exogenous thiols suggests that therapeutic strategies effective in abrogating bladder damage will offer little lung protection.

The last series of experiments tested the hypothesis that the prolonged presence of fibrin clots enhances lung damage. The rationale for these experiments was based on the work of Law (*14*) and Ts'ao and Ward (*15*), who have suggested that vascular damage following lung irradiation promotes exudation of fibrin plugs and that the presence of fibrin promotes the deposition of collagen associated with pulmonary fibrosis. When mice were maintained on daily doses of EACA, no difference in lung lavage protein was seen, compared to that observed in animals treated with Cy only. In addition, no enhancement of lung damage, as measured by breathing frequency, was observed over a range of Cy doses. It is yet to be determined whether the lesion responsible for Cy-induced lung damage is the same as that which produced radiation-induced pneumonitis and fibrosis. Therefore, it is unclear whether a similar lack of enhancement would be observed in radiation-treated animals maintained on antifibrinolytic therapy. Nonetheless, these results suggest that strategies aimed at manipulating vascular permeability may offer little protection for patients receiving large doses of Cy.

REFERENCES

(*1*) Trask CWL, Joannides T, Harper PG, et al: Radiation-induced lung fibrosis after treatment of small cell carcinoma of the lung with very high-dose cyclophosphamide. Cancer 55:57–60, 1985.

(*2*) Buckner CD, Meyers JD, Springmeyer SC, et al: Pulmonary complications of marrow transplantation—Review of the Seattle experiment. Exp Hematol 12(suppl 15):1–5, 1984.

(*3*) Weiner RS, Bortin MM, Gale RP, et al: Risk factors associated with interstitial pneumonitis following allogeneic bone marrow transplantation for leukemia. Transplantation Proc 17:470–474, 1985.

(*4*) Siemann DW, Macler L, Penney DP: Cyclophosphamide-induced pulmonary toxicity. Br J Cancer 53(suppl VII):343–346, 1986.

(*5*) Travis EL, Vojnovic B, Davies EE, et al: A plethysmographic method for measuring function in locally irradiated mouse lung. Br J Radiol 52:67–74, 1979.

(*6*) Lowry O, Rosenbrough H, Farr A, et al: Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275, 1951.

(*7*) Collis CH, Wilson CM, Jones JM: Cyclophosphamide-induced lung damage in mice: Protection by a preliminary dose. Br J Cancer 41:901–907, 1980.

(*8*) Batist G, Andrews JL: Pulmonary toxicity of antineoplastic drugs. JAMA 246:1449–1453, 1981.

(*9*) Travis EL, Meistrich ML, Finch-Neimeyer MV, et al: Late functional and biochemieal changes in mouse lung after irradiation: Differential effects of WR-2721. Radiat Res 103:219–231, 1985.

(*10*) Gurtoo HL, Berrigan MJ, Love J, et al: Metabolism-dependent toxicities of cyclophosphamide and protection by *N*-acetylcysteine and other thiols. *In* Experimental and Clinical Progress in Cancer Chemotherapy. Boston: Martinus Nijhoff, 1985, pp 61–79.

(*11*) Scheef W, Klein HO, Brock N, et al: Controlled clinical studies with an antidote against the urotoxicity of oxazaphosphorines: Preliminary results. Cancer Treat Rep 63:501–505, 1979.

(*12*) Botta JA Jr, Nelson LW, Weikel JH Jr: Acetylcysteine in the prevention of cyclophosphamide-induced cystitis in rats. J Natl Cancer Inst 51:1051–1058, 1973.

(*13*) Berggren M, Dawson J, Moldeus P: Glutathione biosynthesis in the isolated perfused rat lung: Utilization of extracellular glutathione. FEBS Lett 176:189–192, 1984.

(*14*) Law MP: Vascular permeability and late radiation fibrosis in mouse lung. Radiat Res 103:60–76, 1985.

(*15*) Ts'ao C-H, Ward WF: Plasminogen activator activity in lungs and alveolar macrophages in rats exposed to graded single doses of γ-rays to the right hemithorax. Radiat Res 103:393–402, 1985.

Effect of Continuous Infusion of Bleomycin on Repopulation in Mouse Lip Mucosa During Fractionated Irradiation[1]

L. Vanuytsel,* Y. Feng, W. Landuyt, and E. van der Schueren[2,3]

ABSTRACT—**The effect of the administration of bleomycin on repopulation in mouse lip mucosa during fractionated irradiation was studied. Two equal-sized irradiation fractions were delivered with 1-, 7-, or 10-day intervals, with and without simultaneous administration of 40 mg bleomycin/kg in a continuous sc infusion, given over 7 days. In the experiments with irradiation only, the prolongation of the interval from 1 day to 7 days resulted in a net increase of the isoeffective dose due to compensatory proliferation. This effect was even more pronounced with the 10-day treatment interval. The addition of bleomycin, however, nearly completely suppressed this proliferative activity.—NCI Monogr 6:55-58, 1988.**

Repopulation due to accelerated proliferation of stem cells can occur in rapidly regenerating tissues, such as skin and mucosae, during protracted irradiation treatments. It is an attempt of the tissue to restore the radiation-induced cellular depletion, and it will reduce the final damage level. Experimental data indicate that repopulation is already measurable in mouse lip mucosa 3 to 4 days after the start of treatment (*1*) and in mouse skin at about days 10 to 14 (*2,3*). A rapid onset of repopulation with a progressive increase of proliferative activity the following days was also observed in human oropharyngeal mucosae and skin (*4*).

In several clinical studies, radiation and concomitant administration of bleomycin was used for the treatment of patients with head and neck cancer or esophageal cancer. The results of these studies pointed out the increased severity of the mucosa and skin reactions, necessitating reductions of the total radiation doses (*5*) or temporary (*6,7*), or even permanent (*7*), interruption of treatment.

In previous irradiation experiments, using the mouse lip musoca model (*8*), we demonstrated a dose-modifying effect of bleomycin, probably due to interference with accumulation or repair of sublethal radiation damage on top of an independent cell kill (*9,10*).

In the present study, we have tried to assess separately the interference of bleomycin with repopulation, which occurs during protracted irradiations. This could also be a contributing factor in the enhanced mucosal reactions.

MATERIALS AND METHODS

The mice used were female and were obtained from the Naval Medical Research Institute (Bethesda, MD), weighing 23 to 25 g at the time of treatment. They were kept in conventional housing, with free access to food and water.

For a good positioning during irradiation, we anesthetized the animals with enflurane, using a semiclosed inhalation system (*11*). The technique of local irradiation of the lip mucosa has been described previously (*8*). Irradiations were performed with a cobalt-60 source, at a dose rate of 1.4 Gy/minute and a source to skin distance of 60 cm. Twenty-four mice, 6 per dose point, were irradiated simultaneously.

Bleomycin was dissolved in sterile 0.9% saline immediately before use. This solution is stable for at least 7 days at 37° C (*12*). It was administered via osmotic minipumps implanted subcutaneously on the back of the animals. The pumps have a constant volume delivery rate during the first 7 days, after which they were removed (*13*). For all experiments, a total dose of 40 mg/kg was used. This dose, on its own, results in a maximum weight loss of 5% to 6%, with no deaths observed during the first 30 days after the implant (*6*).

The scoring system used is a combination of two arbitrary scales, one for edema and one for reddening, exudation, and crusting (*8*) as shown in table 1. The scoring was done every day by at least two observers over a period of up to 30 days after the irradiation. The mice were killed when they reached the scoring level of 5 without edema. Toxicity was also assessed by daily weight measurements, and the animals were killed when a weight loss of 35% or more occurred.

For each radiation dose we constructed a reaction curve by plotting the mean daily score of all animals as a function of time after the first irradiation. The mean peak value was plotted against the total radiation dose for the dose-response curves. To compare the various treatment regimens, we selected the mean peak reaction level 3.5 (isoeffect).

Experiments were performed with 2 equal-sized fractions separated by either 1, 7, or 10 days. The irradiations in the combination experiments with 1- and 7-day intervals were timed in such a way that the pumps were removed 4 hours after the second irradiation. Thus all irradiations were performed during the infusion treatment. In the 10-day interval experiment, the first irradiation was given 2 days before the implants and the second fraction 1 day after the removal of the pump. All experiments were repeated at least twice.

---

[1] Supported by a grant of the Algemene Spaar- en Lijfrentekas of Belgium.

[2] Department of Experimental Radiotherapy, University Hospital St. Rafael, Leuven, Belgium.

[3] We thank M. Ramaekers for technical assistance and L. Minnen for secretarial assistance.

**Reprint requests:* L. Vanuytsel, M.D., Department of Experimental Radiotherapy, University Hospital St. Rafael, Capucijnenvoer 33, 3000 Leuven, Belgium.

TABLE 1.—Scoring system for the mouse lip mucosa reaction

Score	Observation
0.5	Doubtful if abnormally pink
1	Slight but definite reddening
2	Severe reddening
3	Focal desquamation
4	Crusting or exudation covering about one-half the lip area
5	Crusting or exudation covering more than one-half of the lip area
	Separate scores for edema (swelling) of the lips are to be added to the above score, giving a maximum score of 7.
0.5	Doubtful if any swelling
1	Slight but definite swelling
2	Severe swelling

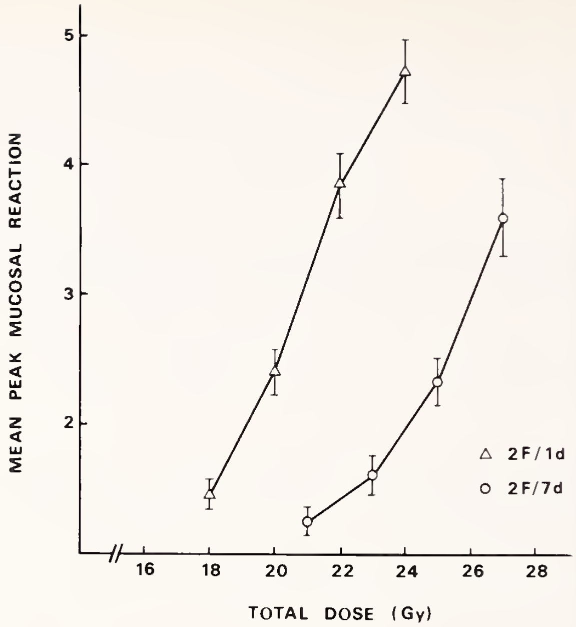


FIGURE 1.—Dose-response curves showing the mean peak mucosal reactions of mice treated with 2 equal-sized irradiations with either 1 day (△) or 7 days (○) between treatments.

RESULTS

The mucosal reaction curves obtained with experiments using 2 radiation fractions with either 1- or 7-day intervals are monophasic (data not shown). There is no difference in the time course of development of peak mucosal reactions for these 2 irradiation schedules. The dose–response curves from these experiments are shown in figure 1. The curve for the experiment with a 7-day interval between irradiations is shifted to higher total radiation doses compared with the curve from the experiment with a 1-day interval. The isoeffective doses, at scoring level 3.5, are 21.5 Gy and 26.9 Gy, respectively, for the 1- and 7-day intervals, i.e., an increase in the isoeffective dose of about 5 Gy. This increase is largely due to the sparing effect of compensatory proliferation. With a radiation interval of 10 days, the mucosal reaction curves become biphasic because of the large amount of repopulation between the 2 irradiation sessions [fig. 2, (*1*)]. Therefore, we made no attempt to construct a dose–response curve because it would give an underestimate of the reaction.

When the 2 irradiations with either 1- or 7-day intervals were given during a 7-day continuous infusion of bleomycin, the resulting dose–response curves were as shown in figure 3. No shift to higher radiation doses of the 7-day interval curve is observed. The two dose–response curves are completely superimposing, with isoeffective doses (at level 3.5) of 17.8 Gy and 18.1 Gy for the 1- and 7-day intervals, respectively. These data suggest that no measurable repopulation occurred during the longer interval. The mucosal reaction curve for the 10-day interval in the combined treatment remains monophasic (fig. 4), which also indicates a considerable inhibition of proliferation. Because in the latter experiment the irradiations were not performed during the drug infusion, the impact of other factors contributing to the severity of the mucosal reaction [such as interaction with sublethal damage repair (*10*)] can be different in this experiment, compared with the other ones. Therefore, we thought it inappropriate to compare the isoeffective doses of this experiment with those of the 1- and 7-day interval treatment.

DISCUSSION

Repopulation plays an important role in reducing the reactions of rapidly proliferating tissues to a protracted,

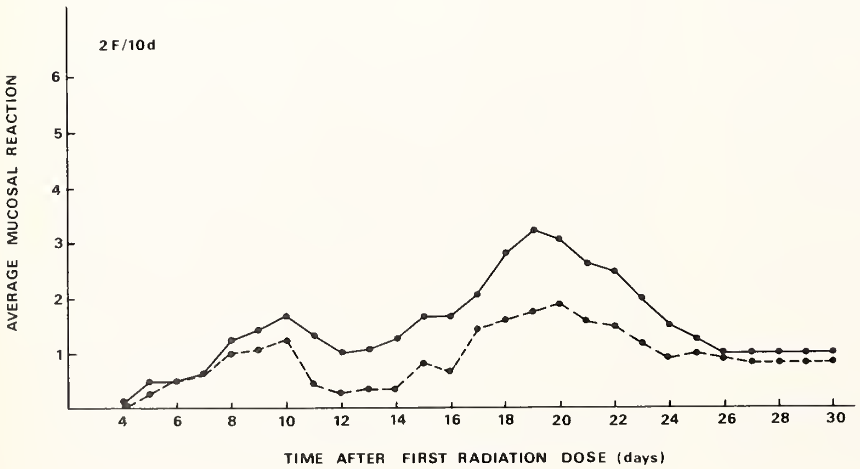


FIGURE 2.—Average acute lip mucosa reactions following 2 equal-sized radiation fractions separated by 10 days, plotted as a function of time after the first irradiation. Curves refer to fraction sizes of 13.5 Gy (- - -) or 14.5 Gy (——).

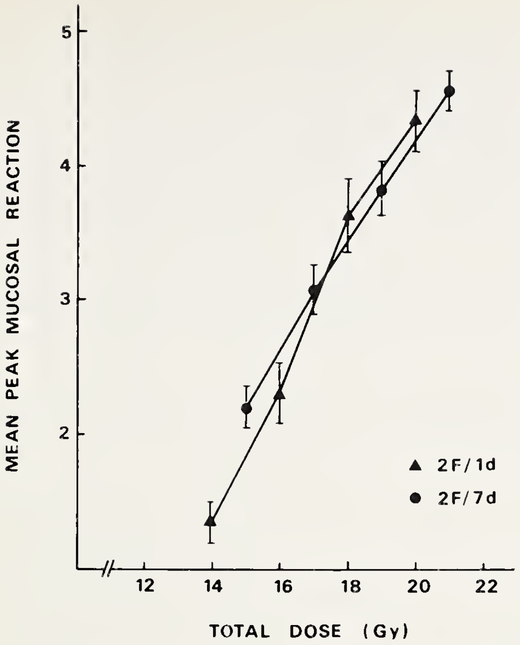


FIGURE 3.—Dose-response curves after 2 equal-sized radiation doses separated by either 1 day (▲) or 7 days (●). Irradiations were delivered during a continuous sc infusion of 40 mg bleomycin/kg for 7 days.

fractionated irradiation treatment. This is clearly shown in several clinical studies on head and neck cancer with fractionation schedules concentrated in time, in which the possibility of repopulation was strongly reduced. The reported mucosal reactions were more severe than observed after conventional prolonged treatments (*4*).

The impact of repopulation on the final outcome of a protracted irradiation treatment on mucosal tissues is also clear from the present experiment on mouse lip. Prolongation of the overall treatment time from 1 to 7 days resulted in an increase of the isoeffective dose of about 5 Gy. The simultaneous administration of bleomycin, however, completely eliminated the need for increasing the radiation dose, with prolongation of treatment time, which indicated that repopulation was largely suppressed during the bleomycin infusion. A reduction of the compensatory proliferation was also reported in the same mouse model when dactinomycin was given between subsequent irradiations (*14*).

In previous studies on mouse lip mucosa, using the combination of bleomycin and irradiation, we observed an interference of the drug with the cellular capacity to accumulate or to repair sublethal radiation damage, in addition to an independent cytotoxic effect of both agents (*9,10*). Furthermore, the present results show a definite interference of the drug with repopulation occurring after irradiation. Thus it becomes increasingly clear that a drug, when administered during a radiation treatment, can interfere at the same time with several mechanisms of recovery from radiation damage. Therefore, it is very important that researchers investigate and quantify separately these possible mechanisms of interference before combining irradiation with a cytotoxic drug treatment or before changing the irradiation fractionation schedule in an established combination treatment.

REFERENCES

(*1*) ANG KK, XU FX, VANUYTSEL L, et al: Repopulation kinetics in irradiated mouse lip mucosa: The relative importance of treatment protractions and time distribution of irradiations. Radiat Res 101:162–169, 1985.

(*2*) ANG KK, LANDUYT W, RIJNDERS A, et al: Differences in repopulation kinetics in mouse skin during split course multiple fractions per day (MFD) or daily fractionated irradiations. Int J Radiat Oncol Biol Phys 10:95–99, 1984.

(*3*) DENEKAMP J: Changes in the rate of repopulation during multifractionation irradiation of mouse skin. Br J Radiol 46:381–387, 1973.

(*4*) VAN DER SCHUEREN E, VAN DEN BOGAERT W, ANG KK: Radiotherapy with multiple fractions per day (MFD). *In* Biological Basis of Radiotherapy (Peckman M, Adams G, Steel G, eds). Amsterdam: Elsevier, 1983, pp 195–210.

(*5*) TANAKA Y, WADA T, FUCHIHATA H, et al: The combined treatment with radiation and bleomycin for intra-oral carcinoma. A preliminary report. Int J Radiat Oncol Biol Phys 1:1189–1193, 1976.

(*6*) SEAGREN SL, BYFIELD JE, NAHUM AM, et al: The treatment of locally advanced squamous cell carcinoma of the head and neck with concurrent bleomycin and external beam radiation therapy. Int J Radiat Oncol Biol Phys 5:1531–1535, 1979.

(*7*) CACHIN Y, JORTAY A, SANCHO H, et al: Preliminary results

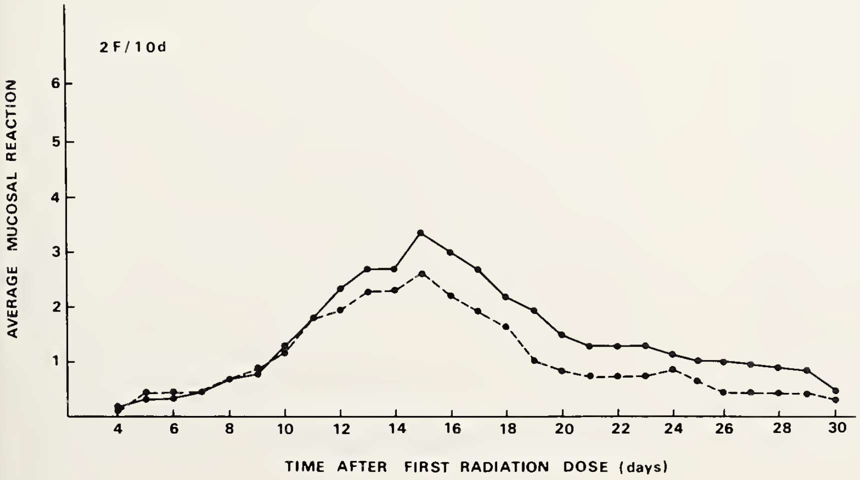


FIGURE 4.—Acute lip mucosal reactions following treatment with 2 equal-sized irradiations (10-day interval) in combination with sc bleomycin infusion (40 mg/kg) for 7 days starting at 2 days after the first radiation dose. Curves refer to fraction sizes of 10 Gy (---) or 11 Gy (——).

of a randomized EORTC study comparing radiotherapy and concomitant bleomycin to radiotherapy alone in epidermoid carcinomas of oropharynx. Eur J Cancer 13: 1389–1395, 1977.

(*8*) Xu FX, van der Schueren E, Ang KK: Acute reactions of the lip mucosa of mice to fractionated irradiation. Radiother Oncol 1:369–374, 1984.

(*9*) Feng Y, Vanuytsel L, Landuyt W, et al: The combined effect of bleomycin and irradiation on mouse lip mucosa. I. Influence of timing, sequence and mode of drug administration with single dose irradiation. Radiother Oncol 6:143–151, 1986.

(*10*) Vanuytsel L, Feng Y, Landuyt W, et al: The combined effect of bleomycin and irradiation on mouse lip mucosa. II. Influence on the accumulation and repair of sublethal damage during fractionated irradiation. Radiother Oncol 6:267–273, 1986.

(*11*) Ang KK, van der Kogel AJ, van der Schueren E: Inhalation anesthesia in experimental radiotherapy: A reliable and time-saving system for multifractionation studies in a clinical department. Int J Radiat Oncol Biol Phys 8:1665–1670, 1982.

(*12*) Sikic BI, Collins JM, Mimnaugh EG, et al: Improved therapeutic index of bleomycin when administered by continuous infusion in mice. Cancer Treat Rep 62:2011–2017, 1978.

(*13*) Theeuwes F, Yum SI: Principles of the design and operation of generic osmotic pumps for the delivery of semisolid or liquid drug formulations. Ann Biomed Eng 4:343–353, 1976.

(*14*) Landuyt W, van der Schueren E, Ang KK: The effect of actinomycin D on radiation induced reactions of the lip mucosa of mice. Int J Radiat Oncol Biol Phys 11:1503–1512, 1985.

Rodent Model of Chemoradiotherapy-induced White Matter Necrosis [1]

Eugene M. Taylor,[2,3] J. Russell Geyer,[2,3] Jerrold M. Milstein,[2,3] Cheng Mei Shaw,[3] Joseph P. Geraci,[4] Peter Wootton,[3] Bridgette A. Hubbard,[2] and W. Archie Bleyer[2,3,5,*]

ABSTRACT—**This report describes a laboratory model that permits study of the radiochemotherapy interactions in the CNS. Rats are stereotaxically implanted with a cerebroventricular cannula attached to an osmotic minipump, which slowly infuses a chemotherapeutic agent into CSF for up to 14 days. The cervical cord is irradiated, and forelimb paralysis develops 4–6 months later at an effective dose for paresis in 50% of the animals; the doses with radiotherapy alone are 2,125 cGy for a single fraction and 2,950 cGy for split fractions. Investigations with the model indicate that mature CNS tissue is not sensitized to either single-fraction or split-dose irradiation with either simultaneous or post-radiation exposure to high concentrations of methotrexate.—NCI Monogr 6:59–64, 1988.**

An increasing number of patients with leukemia, non-Hodgkin's lymphoma, CNS tumors, and brain and spinal cord metastases are being treated with chemoradiotherapy directed at the CNS. Patients with these malignancies represent approximately one-half of all children with cancer and >10% of adults with cancer. When either of the two commonly used intrathecal chemotherapy agents MTX or cytarabine is used with CNS irradiation, the therapy-limiting toxicity is white matter necrosis, either leukoencephalopathy or LMP, depending on which part of the CNS is irradiated (*1–7*).

The pathogenesis of the sequelae of radiotherapy and chemotherapy directed to the CNS is poorly understood (*2,3*). Because of confounding variables, resolution of this problem by retrospective study of clinical experience is unlikely to yield satisfactory information. Similarly, prospective clinical studies would be faced with formidable problems of ethics, design, and interpretation. Therefore, for the past 5 years, we have been developing a rat model of white matter necrosis that permits study of the interactions between radiotherapy and chemotherapy and that may help to determine the pathogenesis and prevention of adverse chemoradiotherapy interactions.

METHODS

Animals

The model was developed with adult female rats 12–16 weeks of age and weighing 250–300 g. Sprague-Dawley rats from Tyler Laboratories (Bellevue, WA) are used, and all animals are of the same sex, to minimize possible sex-related variables. After undergoing surgery or other experimental treatment, the animals are maintained in separate cages on wire-grid floors over pelletized cellulose bedding. They are fed Purina laboratory chow and water ad libitum.

Anesthesia

A number of anesthetic regimens were evaluated with respect to the special requirements of this set of studies, particularly the need for repeated anesthetic administration in animals receiving potentially hepatotoxic and nephrotoxic drugs. A mixture of ketamine (36 mg/kg) and xylazine (3.6 mg/kg) administered im was used for all procedures lasting more than a few minutes, and methoxyflurane given by mask was selected for brief procedures such as blood drawing and removal of pumps.

Implantation of Pumps

Miniature osmotic pumps (Alzet Minipump Model 2002; Alza Corp., Palo Alto, CA) are implanted in rats that are to receive drug or saline-control infusions. The pump is 3.0×0.7 cm in external dimensions and has a reservoir capacity of 200 μl. A minipump can hold up to 2 mg of MTX, and in situ, each pump is designed to infuse a solution at a constant rate of 0.5 μl/hour for 14 days. Our in vitro pump outflow measurements and in vivo pharmacologic determinations verify the performance of the pump as specified by the manufacturer.

Pumps are implanted sc in the interscapular fossa using blunt dissection to create an sc pocket. The calvarium is exposed, and using the bregma as a reference point, a hole is drilled (No. 68 drill) for the cannula at a point 0.5 mm posterior to the coronal suture and 1.5 mm lateral to the sagittal suture. A second hole is drilled (No. 73 drill) in the

---

ABBREVIATIONS: MTX = methotrexate; LMP = leukomyelopathy; MTD = maximal tolerated dose; $LD_{5/30}$ = lethal dose for 5% of animals by 30 days after pump insertion; $ED_{50/210}$ = effective dose for paresis for 50% of animals by 210 days after irradiation.

---

[1] Supported by Public Health Service grant CA-31351 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and the American Cancer Society (Washington Division). W. A. Bleyer is an American Cancer Society Professor of Clinical Oncology, University of Washington School of Medicine, Seattle.

[2] Department of Medicine, Jennifer Sacco International Research Laboratories, Children's Hospital and Medical Center, Seattle, WA.

[3] Departments of Pediatrics (*E. M. Taylor, J. R. Geyer, J. M. Milstein,* and *W. A. Bleyer*), Radiation Oncology (*P. Wootton*), Neurology (*J. M. Milstein*), and Pathology (*C. M. Shaw*), University of Washington School of Medicine, Seattle.

[4] Department of Radiological Sciences, University of Washington School of Public Health, Seattle.

[5] Department of Pediatrics, Fred Hutchinson Cancer Research Center, Seattle, WA.

* *Reprint requests:* W. Archie Bleyer, M.D., P.O. Box C-5371, Seattle, WA 98105.

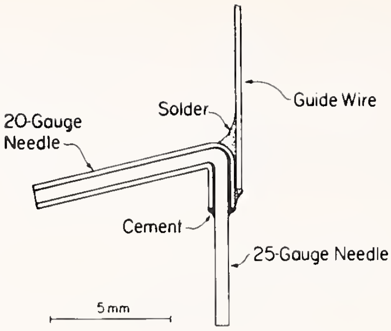


FIGURE 1.—Intraventricular cannula is from 25-gauge hypodermic needle, and hub is from 20-gauge needle. After ventricular insertion, cannula is attached to osmotic pump via polyethylene tubing.

contralateral parietal bone for a stainless-steel screw (000-120 × ⅛ inch). The tip of the stainless-steel cannula (fig. 1) is placed in the right lateral ventricle, using standard stereotaxic technique, and is connected to the minipump with a polyethylene (PE 60) catheter. The anchor screw, cannula, and catheter are then cemented to the cranium with dental acrylic cement, and the skin incision is closed with nylon suture.

Irradiation

Ketamine/xylazine anesthesia provides rapid immobilization and early recovery after completion of radiotherapy. The rats are placed 2 at a time into a Plexiglas holder and irradiated in a dorsoventral direction (fig. 2). A lead collimator restricts radiation to a 1.5-cm segment of the cervical spinal cord. Rats are positioned in the jig to limit radiation to the portion of spinal cord between the occipital crest and the second thoracic vertebra.

Photon radiation is delivered from a cesium-137 source in a dedicated small animal irradiator located at the University of Washington. Dosimetric studies with lithium fluoride rods implanted in rats that are killed indicate that the mean dose rate is 146 cGy/minute.

Blood and Cerebrospinal Fluid Collection and Methotrexate Analysis

Rats are anesthetized with methoxyflurane by mask for collection of blood samples. Blood is drawn from the retro-orbital plexus with Pasteur pipettes. Aliquots of blood are transferred to serum-separator Microtainers (Becton Dickinson, Rutherford, NJ), which are centrifuged for 3 minutes at 15,000 rpm; the serum is frozen at −4° C for later analysis. The CSF is collected by cisternal puncture, which is performed with a 30-gauge needle, using stereotaxic instrumentation. This method allows the reliable collection of 50 μl of CSF.

The MTX levels are determined by the dihydrofolate reductase inhibition method. This assay measures unchanged MTX but not 7-hydroxymethotrexate or diaminomethylpteroic acid. Brain MTX is measured with a method we have published (*8*).

Determination of Maximal Tolerated Dose

To maximize the efficacy of the model, we attempted to determine the MTD of intraventricular MTX used alone.

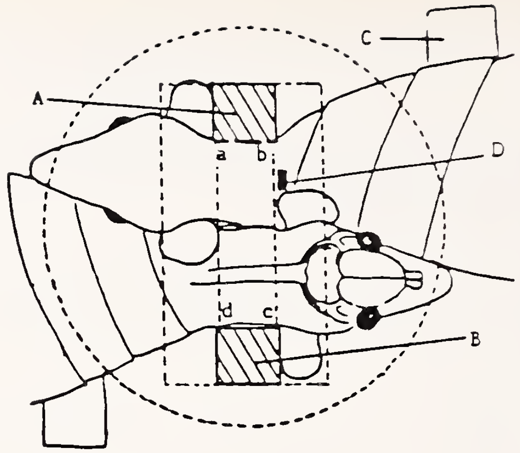


FIGURE 2.—Dorsal view of arrangement for irradiation of cervical cord. Two anesthetized rats are positioned as shown between acrylic posts A and B and are secured with tape C. Prominent spine of 2nd thoracic vertebra D defines posterior border of field. *Dotted lines* indicate position of collimator, which rests on acrylic posts. Aperture that determines field boundaries is rectangle a-b-c-d. Lower rat shows orientation of CNS structures in irradiated field.

We anticipated that when radiation was combined with MTX, there would be increased mortality from acute complications of the pharyngitis and esophagitis that occur after cervical cord irradiation. Thus, we designated the MTD of MTX alone as the $LD_{5/30}$. We expected that this dose would maximize the anticipated delayed neurotoxic effects and yet allow 95% of the animals to survive the acute toxic effects of MTX.

An MTD for intraventricular MTX was determined by varying the pump concentration of MTX from 6.1×10^{-4} to 9.3×10^{-3} *M*. This resulted in plasma MTX concentrations from 10^{-9} to 10^{-7} *M* (fig. 3, *left*). The steady-state plasma levels were directly proportional to the pump concentrations (fig. 3, *right*). In these preliminary studies, the expected systemic MTX toxic effects of mucositis, myelosuppression, weight loss, and gastrointestinal hemorrhage were observed. The $LD_{5/30}$ for MTX alone, expressed as the concentration of MTX in the minipump used to infuse the drug intraventricularly for 14 days, was 4×10^{-3} *M* (fig. 4). The $LD_{5/30}$ MTX dose pump concentration of 4×10^{-3} *M* was used in all succeeding experiments.

Evaluation of Drug and Radiation Effects

Animals are maintained for a minimum of 7 months to allow for the development of forelimb paralysis, the clinical end point of the radiation toxicity. More than 90% of the animals that develop paralysis do so between 120 and 180 days after irradiation. The earliest paralysis is observed at 80 days, and <4% of the paralyzed animals develop this complication between 80 and 120 days. When paralysis occurs in our model, it occurs rapidly, over 12–24 hours. In most animals, complete bilateral paralysis usually occurs within a day after apparently normal forelimb function.

In experiments investigating the combined effects of MTX and radiation, the radiation was administered 7 days prior to a 14-day infusion of MTX, midway through the MTX infusion (i.e., day 7 of the 14-day infusion), or 7 days

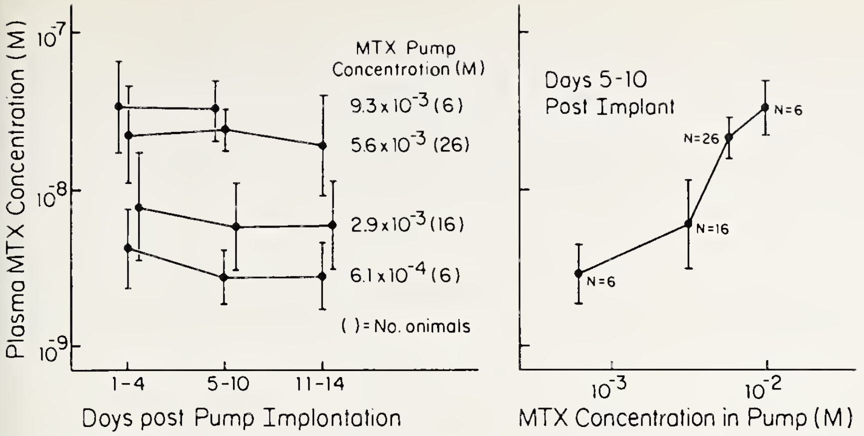


FIGURE 3.—Plasma MTX levels during 14-day intraventricular infusions. (*Left*) Levels during infusion appear to reach steady state within 1–4 days and maintain steady state for remainder of 14 days, at each of 4 pump doses studied. (*Right*) Correlation between steady-state plasma MTX level and pump concentration appears to be linear over range of pump-loading doses studied.

after the end of the infusion (i.e., 21 days after the start of the 14-day infusion). In "split" radiation experiments, the total dose was divided into two equal parts that were administered 24 hours apart on days 7 and 8 of the 14-day drug infusion.

With the development of paresis or at the end of 7 months, each rat is anesthetized with sodium pentobarbital and is then perfused via the left cardiac ventricle with saline, followed by neutral phosphate-buffered Formalin. The brain including the cerebrum, cerebellum, and brain stem is cut coronally into sections 2–3 mm thick, and the cervical spinal cord is cut horizontally into sections 2–3 mm thick. The sections are embedded in paraffin blocks and then cut into sections 5–6 μm thick.

Using Luxol fast blue and Weigert and Holzer stains, we observed three major types of lesions: 1) spongy degeneration with swelling of axons and rarefaction of myelin; 2) coagulative necrosis with foci of eosinophilic coagulum and nuclear debris; and 3) cystic necrosis with liquefaction of necrotic coagulum. The three types of lesions appear to represent different phases of the same process, and therefore we grade the severity of LMP damage in the range of 0–3 on the basis of the lesion density (table 1).

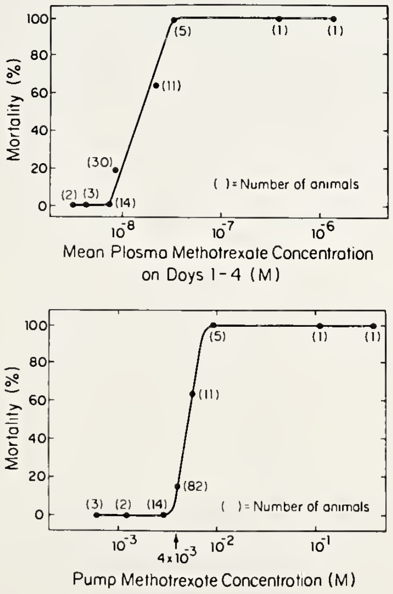


FIGURE 4.—Mortality as function of pump and plasma MTX concentrations. (*Upper*) Mortality vs. plasma concentration. (*Lower*) Mortality vs. pump concentration, with $LD_{5/30}$ at 4×10^{-3} *M*.

RESULTS

To date, >1,000 rats have been used in various applications of these methods. Initial studies using pumps filled with Evans-blue dye verified successful intraventricular cannulation with >95% accuracy, and subsequent monitoring of catheter placement continues to confirm the reliability of this technique.

Radiation.—A steep dose-response curve for single-fraction radiation was observed. All animals that received doses ≤2,000 cGy remained free of paresis for ≤8 months after irradiation, and all animals treated with ≥2,200 cGy demonstrated paralysis (fig. 5). The $ED_{50/210}$ was 2,125 cGy. When radiation was delivered in two fractions separated by 24 hours, the $ED_{50/210}$ was 2,950 cGy (fig. 5). The amount of recovered dose was 825 cGy, which is equivalent to a fractional repair value of 0.56. In each case, the $ED_{50/210}$ value is defined as the intersect of the dose-response curve.

Concordance between clinical and histopathological radiotoxicity.—All animals with paralysis were killed immediately after the cervical cord dysfunction was observed. Animals not demonstrating paralysis were killed 7 months after irradiation, as described previously. The concordance between premortem paralysis and postmortem white matter necrosis is shown in table 2 for all 130 animals examined (C. M. Shaw); examination was performed without knowledge of the premortem status. Overall, there was an 85.4% concordance, and if the 1 animal with questionable paresis and the 17 animals with grade 1 LMP were excluded, the concordance was 90.2%. There was a better concordance when grade 1 LMP was included with grade 2–3 LMP (86.0%) than when it was included with grade 0 LMP (83.1%). In addition, the majority (10 of 17) of the animals with grade 1 LMP had antemortem paralysis.

TABLE 1.—Criteria for grading severity of LMP (white matter necrosis)

Grade	Criteria
0	No necrosis in spinal cord
1	White matter necrosis in 1 section of cord
2	Necrotic lesions in >1 section but not in all sections
3	Lesions of white matter necrosis in all cord sections

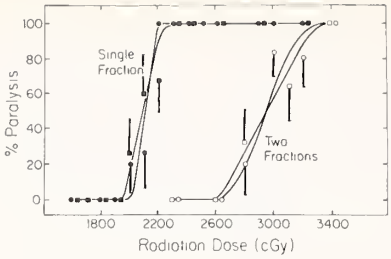


FIGURE 5.—Paralysis-dose response for single- and split-fraction radiotherapy with (*squares*) and without (*circles*) simultaneous MTX. Each *square* and *circle* represents 6 animals. The studies with single-fraction radiotherapy were performed with radiation alone or with radiation on 7th day of 14-day MTX infusion. Studies with split-fraction radiotherapy were conducted with radiation alone or with radiation on the 7th and 8th days of 14-day MTX infusion. Dose shown is sum of 2 fractions. *Vertical bars* designate ±1 SE of proportion.

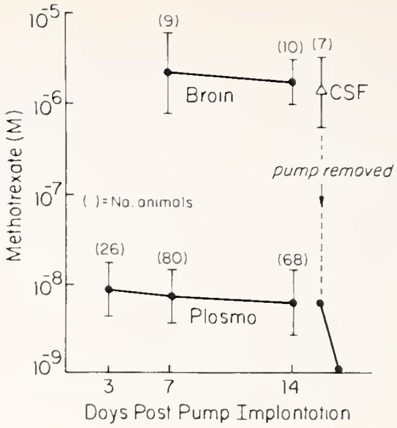


FIGURE 6.—MTX concentrations in CSF, plasma, and brain during 14-day intraventricular infusions in nonirradiated animals. *Vertical bars* designate ±2 SD values.

Pharmacology and neurotoxicity of intraventricular methotrexate.—Plasma levels during the 14-day MTX infusions in nonirradiated animals given the $LD_{5/30}$ dose ranged between 0.5 and 2.0×10^{-8} *M* (fig. 6). The mean CSF concentration was $1.6\pm2.5\times10^{-6}$ *M* (mean ± SD); the mean simultaneous plasma concentration was $1.5\pm5.8\times10^{-8}$ *M*; and the mean CSF to plasma ratio was 73 ± 84. In general, the CSF to plasma drug ratios achieved in the rat are similar to those observed in children and adult patients (*9*). The mean whole brain concentrations were $2.4\pm3.3\times10^{-6}$ *M* at 7 days and $1.8\pm1.3\times10^{-6}$ *M* at 14 days (fig. 6); the mean brain to plasma ratio was 138. Disappearance of MTX from plasma after pump removal was rapid, with concentrations $<10^{-9}$ *M* observed within 24–72 hours. There was no discernible spinal cord toxicity, either clinical (paralytic) or histopathological (LMP), in any of the animals that were given MTX intraventricularly for 14 days at any dose.

Deaths from acute complications.—Of the rats treated with radiation alone, 13% died of acute complications, defined as occurring within 30 days of irradiation. When animals were treated with both cervical irradiation and MTX infusions, 19% died within 30 days after irradiation and 21% died within 60 days. Thereafter, deaths were rarely observed until day 80, when the first animals died of paraplegia as a late effect of radiation.

Methotrexate-radiation interactions.—When MTX was given during or after irradiation, there was no effect on the dose-response curve of radiation-induced paralysis with either single-dose or fractionated irradiation; in both cases, the $ED_{50/210}$, which was determined by the intersect of the curves was unchanged (fig. 7). The fractional repair value was 0.56 with or without MTX. In addition, there was no difference in the severity of LMP found at necropsy, as measured by the LMP grade in animals treated with MTX either during or after irradiation, compared with animals that did not receive MTX (data not shown).

TABLE 2.—Clinical (paralytic) vs. histopathological (LMP) toxicity[a]

Toxic effect	Total	LMP grade[b] 0	1	2	3
Definite paralysis	64	2	10	40	12
Possible paresis	1	1	0	0	0
No paresis or paralysis	65	49	7	5	4

[a] Values = No. of animals. Data include all irradiated animals with or without MTX.

[b] Severity of white matter necrosis in irradiated spinal cord.

DISCUSSION

With the advent of "prophylactic" CNS therapy, there was a dramatic improvement in the long-term survival of children with acute lymphoblastic leukemia. It is now clear that at least one-half of the children who received the prophylaxis were cured of this otherwise uniformly fatal disease. The CNS treatment regimens used in this breakthrough were either craniospinal radiotherapy or cranial radiotherapy in combination with intrathecal MTX (*10,11*).

Combinations of radiation and MTX have also shown early promise in treatment of brain tumors (*12–14*), which are second only to acute lymphoblastic leukemia as a cause of cancer incidence and mortality in children. In contrast to the success of treatment of acute lymphoblastic leukemia, however, brain tumors continue to respond poorly to treatment (*15*).

Unfortunately, serious adverse effects of therapy have become evident with long-term survival, and neurological

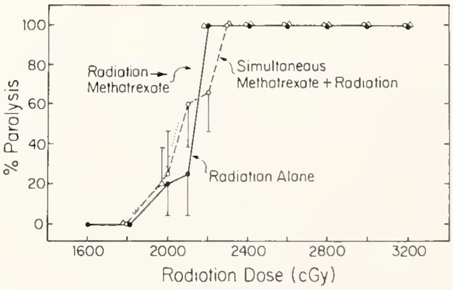


FIGURE 7.—Paralysis-dose response for single-fraction radiotherapy without MTX (*solid circles*) and with MTX administered simultaneously (*open circles*) and after irradiation (*triangles*). *Vertical bars* designate ±1 SE of proportion. Curve for radiation alone is same as shown for single-fraction irradiation in fig. 5.

dysfunction has been reported as the most common of the sequelae in survivors of both acute lymphoblastic leukemia (*16*) and brain tumors. Nearly 20% of survivors of acute lymphoblastic leukemia, for example, have been observed to have evidence of neurological deficits such as leukoencephalopathy, mineralizing microangiopathy, intellectual dysfunction, seizure disorders, and neuroendocrine deficiencies (*16*).

The morbidity associated with CNS cancer therapy has seriously impeded further progress in management of patients with acute lymphoid and CNS malignancies. Reports of leukoencephalopathy following use of radiation and MTX in the treatment of brain tumors have curtailed the development of chemoradiotherapy protocols that might improve the disappointing success rate in management of these diseases.

Reports of specific adverse effects of therapy have been summarized by several investigators (*1–4*). The most serious of these is white matter necrosis (leukoencephalopathy and LMP). Histologically, this condition is characterized by demyelination, beginning with axonal swelling and fragmentation and progressing to coagulative necrosis and gliosis (*1,5–7*). Clinically, the disorder presents as a progressive neurological deterioration, which may continue to ataxia, seizures, hemiplegia, decerebration, and coma (*1,2*). The process may be fatal, but most patients survive with permanent neurological damage. Little is known about the pathogenesis of this condition, except that complex interactions between MTX and radiation appear to be responsible for the majority of cases (*1,2,7*).

The rat is an appropriate animal with which to model white matter necrosis. The animal is small enough to allow economical use in substantial numbers and yet is large enough to allow long-term implantation of cannulas and pumps and to permit injection into and sampling of the CSF. Blood and other tissues can be collected in sufficient quantities for chemical and pharmacologic analysis. Neuropharmacologic data on the rat are extensive; e.g., rat brain permeability constants have been determined for 25 cancer chemotherapeutic agents (*17*). The effects of ionizing radiation on the rodent CNS, particularly the spinal cord, are well documented. As observed in our model, the microscopic appearance of radiation-induced white matter necrosis is identical to that of the lesions observed in patients, as described here (*5–7*). Rat models of leukemia, including CNS leukemia (*18*), have also been developed.

An apparent disadvantage of the rat as a model of leukoencephalopathy is its paucity of cerebral white matter. However, the spinal cord has an equivalent tissue effect, LMP, and this has specific advantages. 1) Paralysis is a more discrete end point than evaluation of a brain function and is less equivocal in interpretation than are subtle cerebral effects. 2) White matter is abundant in the cervical cord of the rat, whereas the central white matter of the brain is relatively small. 3) Local effects of implants in the cerebral ventricles do not obfuscate interpretation of tissue changes in the spinal cord. 4) There is extensive literature on the dose-response relationships in the rat.

Data on aspects of the interaction between MTX and irradiation of the CNS are available in a small number of published animal studies. These include studies of the pharmacokinetics of MTX as affected by CNS radiation in the mouse (*8*) and CSF clearance of MTX following cranial irradiation in the rabbit (*19*). A second group of studies, all using the rat, have specifically addressed the effects of combined MTX and CNS irradiation on cellular pathology (*20*), pathophysiology (*21*), and behavior (*22*). While this set of studies approaches the general problem of MTX-radiation neuropathology, the experiments have been done with single doses of MTX administered by iv, ip, or intralumbar routes. This is unlike the protocols prevailing in clinical practice, which generally use repetitive intra-CSF injections of MTX. Our model was developed to permit investigation of prolonged drug exposures that are more clinically relevant. The results indicate that the model is valid and ultimately can help to determine the pathogenesis and prevention of adverse chemoradiotherapy interactions.

We found no evidence in our model that MTX is a radiosensitizer of the CNS in adult rats. From the split-fraction irradiation experiments, it was apparent that about 56% of the radiation damage from the first dose was repaired by the time the second dose was given. In addition, it appears that MTX does not change this repair fraction in mature neural tissue.

It has also been demonstrated by van der Kogel and Sissingh (*21*) that simultaneous MTX exposure does not radiosensitize mature CNS tissue. Adult WAG/Rij rats treated with orthovoltage radiation to the cervical cord demonstrated an ED_{50} of 2,100 cGy with or without intralumbar MTX at a dose of 4 mg/kg at 0.5–1.0 hour before irradiation (*21*). These findings suggest that adult patients may receive both MTX and either brain or spinal radiotherapy without a deleterious interaction between these two modalities on normal neural tissue.

On the other hand, it is likely that immature neural tissue is vulnerable to adverse MTX-radiation interactions. Yadin and colleagues (*22*) showed that infant rats treated with both MTX and radiation required a greater number of days to learn a task than rats treated with either modality alone. These investigators treated 17-day-old rats with cranial irradiation (1,000 cGy, single dose) and MTX ip 24 hours after irradiation. When the animals were 12- to 14-weeks old, the performance on a simultaneous discrimination task in a standard operant conditioning box was evaluated. Control groups included an untreated group, an undernourished group, and a 30-day-old treatment group, to control for the age factor. Rats given chemoradiotherapy took longer to reach the 80% discrimination criterion, compared to the groups that received single treatment and to the controls (*22*).

Morris and Hopewell (*20*) at the University of Oxford, England, showed radiochemotherapy interactions in a morphological assessment of the developing rat brain. They irradiated the brains of 20-week-old Sprague-Dawley rats with single doses of 400–1,400 cGy with and without MTX at a dose of 4 mg/kg iv at 30–45 minutes prior to irradiation. When rats were killed 6 weeks later, the subependymal plate showed a decrease in the number of cells in mitosis and of small dark nucleated cells that was proportional to the dose of radiation >400 cGy. In the group pretreated with MTX, the shoulder of the dose-response curve was eliminated, and a reduction of 200–330 cGy was required to eliminate one-half of the mitotic figures and one-half of the cells believed to represent the proliferative compartment of the subependymal layer.

We are currently using the model to determine 1) whether immature neural tissue is susceptible to the MTX-radiation interaction, as suggested by numerous clinical observations; 2) whether cytarabine is a potent radiosensitizer of both immature and mature neural tissue; and 3) whether specific antidotes of MTX or radiation can prevent the sequelae without compromising therapeutic benefit.

REFERENCES

(*1*) Bleyer WA: Neurologic sequelae of methotrexate and ionizing radiation: A new classification. Cancer Treat Rep 65(suppl 1):89–98, 1981.

(*2*) Bleyer WA, Griffin TW: White matter necrosis, mineralizing microangiopathy, and intellectual abilities in survivors of childhood leukemia: Associations with central nervous system irradiation and methotrexate therapy. *In* Radiation Damage to the Nervous System (Gilbert HA, Kagen AR, eds). New York: Raven Press, 1980, pp 155–174.

(*3*) Altman AJ, Schwartz AD: The late effects of cancer treatment. *In* Malignant Diseases of Infancy, Childhood and Adolescence, 2nd ed. Philadelphia: Saunders, 1983, p 569.

(*4*) Jaffe N: Late sequelae of cancer therapy. *In* Clinical Pediatric Oncology (Sutow WW, Fernbach DJ, Vietti TJ, eds), 3rd ed. St. Louis: Mosby, 1984, pp 814–816.

(*5*) Bresnan MD, Gilles FH, Lorenzo AV, et al: Leukoencephalopathy following combined irradiation and intraventricular MTX therapy of brain tumors in childhood. Trans Am Neurol Assoc 97:204–206, 1972.

(*6*) Shapiro WR, Chernik NL, Posner JG: Necrotizing encephalopathy following intraventricular instillation of methotrexate. Arch Neurol 28:96–102, 1973.

(*7*) Price RA, Jamieson PA: The central nervous system in childhood leukemia. II. Subacute leukoencephalopathy. Cancer 35:306–318, 1975.

(*8*) Griffin TW, Rasey JS, Bleyer WA: The effect of photon irradiation on blood-brain barrier permeability to methotrexate in mice. Cancer 40:1109–1111, 1977.

(*9*) Bleyer WA, Dedrick RL: Clinical pharmacology of intrathecal methotrexate. I. Pharmacokinetics in nontoxic patients after lumbar injection. Cancer Treat Rep 61:703–708, 1977.

(*10*) Pinkel D: The ninth annual David Karnofsky Lecture. Treatment of acute lymphocytic leukemia. Cancer 43: 1128–1137, 1979.

(*11*) Bleyer WA: Central nervous system leukemia. *In* Leukemia (Gunz F, Henderson ES, eds), 4th ed. New York: Grune & Stratton, 1982, pp 865–911.

(*12*) Broder LE, Rall DP: Chemotherapy of brain tumors. Prog Exp Tumor Res 16:373–399, 1972.

(*13*) Rosen G, Ghavimi F, Nirenberg A, et al: High-dose methotrexate with citrovorum factor rescue for the treatment of central nervous system tumors in children. Cancer Treat Rep 61:681–690, 1977.

(*14*) Djerassi I, Kim JS, Reggev A: Response of astrocytoma to high-dose methotrexate with citrovorum factor rescue. Cancer 55:2741–2747, 1985.

(*15*) Allen JC, Bloom J, Ertel I, et al: Brain tumors in children: Current cooperative and institutional chemotherapy trials in newly diagnosed and recurrent disease. Semin Oncol 13:110–122, 1986.

(*16*) Meadows AT, Krejmas NL, Belasco JB: The medical cost of cure: Sequelae in survivors of childhood cancer. *In* Status of the Curability of Childhood Cancers (Van Eys J, Sullivan MP, eds). New York: Raven Press, 1980, pp 263–275.

(*17*) Levin VA: Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. J Med Chem 23:682–684, 1980.

(*18*) Calvo W, Hoelzer D: Involvement of the central nervous system in rats with acute leukemia L5222. Acta Haematol 55:28–35, 1976.

(*19*) Veninga TS, Dankert J, Ebels EJ, et al: Early transient accumulation of methotrexate in the cerebrospinal fluid of rabbits after treatment with methotrexate and roentgen rays. Acta Radiat Oncol 23:69–73, 1984.

(*20*) Morris AD, Hopewell JW: Combined effects of radiation and methotrexate on the cells of the rat subependymal plate. J R Soc Med 76:848–852, 1983.

(*21*) van der Kogel AJ, Sissingh HA: Effects of intrathecal methotrexate and cytosine arabinoside on the radiation tolerance of the rat spinal cord. Radiother Oncol 4:239–251, 1985.

(*22*) Yadin EL, Bruno L, Micalizzi M, et al: An animal model to detect learning deficits following treatment of the immature brain. Childs Brain 10:376–384, 1983.

Quantitative Risk of Second Cancer in Patients in First Complete Remission From Early Stages of Hodgkin's Disease

Michel Henry-Amar[1,2,*]

For Groupe Pierre et Marie Curie[3] *and the Lymphoma Cooperative Group of the European Organization for Research and Treatment of Cancer*[4]

ABSTRACT—**Thirty-three second cancers, excluding basal cell carcinomas of skin and in situ carcinomas of the cervix uteri, were observed among 1,084 patients in first complete remission from Hodgkin's disease treated from 1964 to 1981 by the Lymphoma Group of the European Organization for Research and Treatment of Cancer and the Groupe Pierre et Marie Curie. Five of these second cancers were acute nonlymphocytic leukemias (ANLL), and five were non-Hodgkin's lymphomas (NHL). The 15-year cumulative proportion was 7.6% for second cancers; 0.7% for ANLL; and 1.2% for NHL. For solid tumors (ST) occurring in a previously irradiated area, it was 1.0% after regional radiotherapy (RT); after extended-field RT, it was 8.2% (P=.009). The relative risk (RR) of ANLL after combined chemotherapy with mechlorethamine, vincristine, procarbazine, and prednisone plus RT (relative to the general population incidence rates) was 39 (P<.001) during the first 4-year period; it was not significantly increased in patients treated by RT without combined chemotherapy. Similar RR was observed for NHL (RR=31; P<.001). Moreover, an increased RR of NHL (RR=53; P<.001) was observed in patients treated by RT without combined chemotherapy after 10 years. For ST, no significant increased risk was observed regardless of the treatment. There is, however, a slight tendency for the risk of ST related to extended-field RT to increase after 10 years.—NCI Monogr 6:65-72, 1988.**

Since patients with early stages of HD can now be cured with a probability that may reach ≥90%, one of the most serious complications of treatment is probably a high risk of developing SC (*1,2*).

Although there is abundant literature on SC occurring after primary HD, quantitative estimates of SC risk are not so common; most are found in studies of short series and/or those with short follow-up (*3*). Moreover, most SC occur after salvage treatment for relapse, and it is difficult to estimate the effect of each type of therapy on risk of SC. We were given the opportunity to analyze the data from four successive controlled clinical trials conducted by the EORTC Lymphoma Cooperative Group and the GPMC. Since most of the patients were in first complete remission from HD and only received initial therapy, our study focuses on quantitative estimates of SC risk with time in relation to initial therapy.

PATIENTS AND METHODS

Patient Population and Treatment

From 1964 to 1981, 1,501 patients with previously untreated upper diaphragmatic early CS of HD were enrolled in four consecutive clinical trials. The study popu-

---

ABBREVIATIONS: HD = Hodgkin's disease; SC = second cancer(s); EORTC = European Organization for Research and Treatment of Cancer; GPMC = Groupe Pierre et Marie Curie; CS = clinical stage(s); ANLL = acute nonlymphocytic leukemia; MOPP = mechlorethamine, vincristine, procarbazine, and prednisone; ST = solid tumors; NHL = non-Hodgkin's lymphoma; VLB = vinblastine; NAT = procarbazine (Natulan); RT = radiation therapy; O = No. of SC observed in patients with HD; E = No. of SC expected in patients with HD; RR = relative risk; ABVD = doxorubicin, bleomycin, vinblastine, and dacarbazine.

---

[1] Département de Statistique Médicale, Institut Gustave Roussy, Villejuif, France.

[2] We thank all the physicians for their participation in data collection; Mrs. N. Dupouy, data manager, Institut Gustave Roussy, for her collaboration; and Dr. M. H. Pejovic and Dr. V. De Gruttola for review of the text.

[3] *Chairman:* B. Hoerni. *Scientific secretary:* H. Eghbali. *Committee of pathologists:* J. Diebold, G. Chomette, and P. Prudhomme de Saint-Maur. *Cooperating centers in France:* Fondation Bergonié, Bordeaux (*H. Eghbali* and *B. Hoerni*); Centre Jean Perrin, Clermont-Ferrand (*C. Dionet*); Hôpital de Compiègne (*D. Zylberait*); Centre René Gauducheau, Nantes (*B. Le Mevel*); Hôpital St. Jacques, Nantes (*J. Rojouan*); Hôtel-Dieu, Paris (*A. Bernadou, C. M. Blanc, D. James,* and *R. Zittoun*); Hôpital St. Antoine, Paris (*A. Audebert, J. Debray, J. Duhamel, M. Krulik,* and *J. Najman*); Hôpital Pitié Salpêtrière, Paris (*J. L. Binet*); Hôpital Tenon, Paris (*A. Laugier* and *C. Le Mir*).

[4] *Chairman:* R. Somers. *Statistician:* M. Henry-Amar. *Scientific secretary:* J. H. Meerwaldt. *Sponsor:* M. Tubiana. *Committee of pathologists:* M. J. J. T. Bogman, B. Caillou, N. Duplay, R. Gérard-Marchant, E. Halkin, P. Van Heerde, R. Heiman, P. M. Kluin, A. M. Mandard, R. Menon, M. F. Prins, J. A. M. Van Unnik, L. Vrints, and C. De Wolf-Peeters. *Cooperating centers in The Netherlands:* Antoni van Leeuwenhoek Ziekenhuis, Amsterdam (*M. Burgers* and *R. Somers*); University Hospital, Leiden (*P. Thomas*); St. Radboud Academic Hospital, Nijmegen (*D. Wagener*); Rotterdamsch-Radiotherapeutisch Instituut, Rotterdam (*J. H. Meerwaldt, M. Qasim, W. Sizoo,* and *B. van der Werf-Messing*); Ziekenhuis Leyenburg, The Hague (*H. Kerkhofs*); and University Hospital, Utrecht (*H. van Peperzeel*). *Cooperating centers in Belgium:* Institut Jules Bordet, Brussels (*J. Henry* and *J. Lustmann Maréchal*); Centre René Goffin, La Louvière (*J. C. Goffin*); Hôpital de Bavière, Liège (*R. Lemaire*); and Academisch Ziekenhuis St. Rafael, Leuven (*E. van der Schueren* and *J. Thomas*). *Cooperating center in Italy:* Istituto di Radiologia, Universita di Firenze (*L. Cionini* and *G. de Guili*). *Cooperating centers in France:* Fondation Bergonié, Bordeaux (*C. Lagarde*); Centre François Baclesse, Caen (*J. Abbatucci* and *A. Tanguy*); Centre Léon Bérard, Lyon (*D. Fière, J. Papillon,* and *J. J. Viala*); Centre Antoine Lacassagne, Nice (*C. Lalanne* and *M. Schneider*); Hôtel-Dieu, Paris (*C. M. Blanc* and *R. Zittoun*); Institut Jean Godinot, Reims (*A. Cattan*); Centre Henri Becquerel, Rouen (*R. Le Fur, M. Monconduit,* and *H. Piguet*); Institut Gustave Roussy, Villejuif (*J. L. Amiel, P. Carde, J. M. Cosset, M. Hayat,* and *M. Tubiana*); and Institut de Cancérologie et d'Immunogénétique, Hôpital Paul Brousse, Villejuif (*G. Mathé*).

* *Reprint requests:* Michel Henry-Amar, M.D., Département de Statistique Médicale, Institut Gustave Roussy, rue Camille Desmoulins, 94805 Villejuif Cedex, France.

Table 1.—Initial treatment and response in study population[a]

Group	Study	Duration of study	No. of patients	In 1st complete remission	Median follow-up (mo)	Treatment
EORTC	H1	1964–1970	373	51%	181	Mantle-field RT ± VLB; 2 yr
EORTC	H2	1972–1976	300	72%	122	Mantle-field + PA ± spleen RT followed by VLB or VLB/NAT; 2 yr for patients with MC–LD histological type
EORTC	H5	1977–1981	494	78%	60	Negative laparotomy: mantle-field RT vs. mantle-field + PA RT Unfavorable prognosis at onset (no laparotomy): total lymphoid RT vs. MOPP × 6 + mantle-field RT
GPMC	H76	1977–1981	335	87%	65	3 or 6 cycles of MOPP + involved-field RT vs. MOPP × 3 or 6 + mantle-field + PA RT
Total			1,501	72%		

[a] PA = para-aortic; MC–LD = mixed cellularity–lymphocytic depletion.

lation is composed of the 1,084 patients (72%) who were still in first complete remission from HD on July 1, 1986 (table 1). The rationales for the different studies as well as inclusion criteria, staging procedures, and treatment types have been previously described (*4–9*). A summary of the modes of therapy used is provided in table 1.

Treatment Categories

There is international consensus that secondary ANLL is related to alkylating agents and combined modality treatments such as MOPP, whereas secondary ST are related to radiation. Moreover, there is some evidence that secondary NHL is more likely to be related to combined modality treatment (*10–13*).

In a previous study, we did not observe any relationship between VLB or VLB/NAT therapy and SC risk (*14,15*). Therefore, patients treated either by RT alone, RT plus VLB, or VLB/NAT were considered as a single group for the risk of ANLL or NHL. The risk in this group was compared to that in a group of patients treated by combination of RT and three or six cycles of MOPP (table 2). In the study relative to secondary ST risk, the extent of irradiated fields was taken into account.

Analysis of Data

The time at risk for SC was computed from the end of initial therapy to the date of death, the date of last known status, or July 1, 1986, whichever came first.

Two approaches were used to estimate the association between treatment and risk. The first method was a comparison of cancer incidence between the population of patients with HD and the general population. In this approach, the exposure to the risk was based on the accumulation of person-years of observation. The ratio of O to E was computed using cancer incidence data from the general population. Expected numbers were computed with the use of cancer incidence rates specific for age, sex, and calendar year that have been published by the Bas-Rhin Tumor Registry in France (*16*) and The Netherlands Central Bureau of Statistics (*17*). The confidence limits of O/E were obtained by assuming the Poisson distribution for O (*18*). This assumption was also used to test the equality of O and E. A one-sided test was used to test RR >1.

Table 2.—Treatment groups

Study	Treatment	No. of patients
ANLL or NHL	RT ± VLB/NAT	668
	RT + MOPP × 3 or 6	416
ST	Regional[a] or involved-field RT ± chemotherapy	534
	Extended-field RT[b] ± chemotherapy	550

[a] Mantle-field RT (complete or incomplete).

[b] Mantle-field + PA RT or total lymphoid RT.

In the second approach, we made comparisons of occurrence of SC within the treatment groups. To compare the occurrence of SC in the 2 treatment groups, we used the Mantel-Haenszel procedure to estimate the cumulative proportion of SC as a function of time since initial treatment (*19,20*). A quantification of the relationship between the time of occurrence of SC and concomitant variables such as age, sex, or CS was calculated using the proportional hazards model described by Cox (*21,22*). In this model, RR of SC can be estimated when adjusted for different follow-up periods.

Statistical comparisons of length of time from date of initial diagnosis to date of last known status were performed by analysis of variance to test the equality of the means and by the Bartlett test to evaluate the homogeneity of variances.

RESULTS

Characteristics of Patients With Second Cancers

The characteristics of the study population are summarized in table 3. The mean age at diagnosis was 31.3 years; 13% of the patients were 40–49 years old, and 9% were ≥50 years. The sex ratio was 1.32; the majority of the patients were males. Of the 1,084 patients, 59% had CS II disease. In H1 and H2 cohorts (table 1), patient characteristics did not differ between treatment groups. Conversely, in cohorts H5 and H76, since these characteristics were used for treatment assignment, patients were younger and more likely to have CS I and to be included in a group that received less aggressive treatment.

The proportion of patients lost to follow-up is low (9%), and these patients generally have been lost after a long period of complete remission (>10 yr).

TABLE 3.—Characteristics of HD population at onset

Characteristic	
Total No. of patients	1,084
Sex ratio	1.32
Age	
Mean (yr)	31.3 (12.1 SD)
40–49 yr	12.5%
≥50 yr	9.1%
CS	
I	41.1%
II	58.9%
Treatment group	
Mantle-field RT	14.0%
Mantle-field RT + VLB	10.7%
Mantle-field + PA ± spleen RT	19.2%
Mantle-field + PA ± spleen RT + VLB	3.8%
Mantle-field + PA ± spleen RT + VLB/NAT	3.9%
Total lymphoid RT	10.0%
MOPP × 3 + involved-field RT	3.5%
MOPP × 3 + mantle-field + PA RT	4.2%
MOPP × 6 + mantle-field RT	11.5%
MOPP × 6 + involved-field RT	9.5%
MOPP × 6 + mantle-field + PA RT	9.7%

Characteristics of Patients with Second Cancers

Thirty-three (3%) of the 1,084 patients developed an SC 10–188 months after initial treatment of primary HD; there was a mean interval of 81 months. The SC population is described in table 4. Patients with basal cell carcinoma of skin (7 patients), in situ carcinoma of cervix uteri (1 patient), or unspecified histological diagnosis (2) were excluded from the present study. Compared to the whole population, the proportion of males was greater among the SC patients (sex ratio = 2.0); the mean age was 42 years (33% ≥50). At the time of diagnosis of SC, 45% were ≥50 years of age; 8 of 10 patients with secondary ANLL or NHL were ≥50 years (table 5). There was no statistically significant difference in mean length of follow-up time between SC patients and the other patients (99 ± 56 vs. 90 ± 51 mo, respectively). The anatomopathological features of the SC cases are given in table 5. Of the 33 patients, 5 had ANLL, 5 had NHL, and 23 had ST. Three of the 5 patients with secondary ANLL were treated by a combination of six cycles of MOPP plus RT to the involved fields; 1 patient received total lymphoid RT; and 1 had mantle-field RT. Two of the 5 patients with secondary NHL were treated by a combination of six cycles of MOPP plus RT; 2 received a combination of extended-field RT plus chemotherapy; and 1 had mantle-field RT plus VLB. Of the 23 ST, 16 were localized in an irradiated area; 13 of these had been initially treated by extended-field RT with or without adjuvant chemotherapy or MOPP. The prognosis of the patients after SC occurred was poor. The 5 patients with ANLL died, as did 4 of the 5 with NHL and 15 of the 23 with ST.

General Population Comparisons

The risks of SC relative to the general population (O/E) were calculated for each type of SC (i.e., ANLL, NHL, or ST localized either in an irradiated or a nonirradiated area) by mode of therapy for the overall population (table 6). Moreover, the RR was calculated within each treatment group by exclusive 5-year intervals.

Risk of Secondary ANLL and NHL

In the overall population, there was excessive occurrence of ANLL (O/E = 16; $P < .001$) and NHL (O/E = 19; $P < .001$). When considering the mode of therapy, we observed a highly significant increase in risk of ANLL in patients treated by combination of MOPP plus RT during the first 4-year period (O/E = 39; $P < .001$). For patients not treated with MOPP, RR was 9.22 ($P = .002$) for the entire group; there was no increase in risk with time.

For patients with NHL, we observed a highly significant increase in risk during the first 4-year period (O/E = 31; $P < .001$) after treatment with a combination of MOPP plus RT and a similar increase in risk after 10 years (O/E = 53; $P < .001$) in those who received RT alone or followed by single-agent chemotherapy.

Risk of Secondary Solid Tumors

No increase in risk of ST localized in a nonirradiated area was observed either in the overall population or in the 2 subgroups of treatment. The same conclusion can be drawn for ST localized in an irradiated area, although a slight but not significant trend to increase with time was observed in patients treated by extended-field RT.

Comparisons Among Treatment Groups

Life-table Analysis

The cumulative proportion of SC is plotted in figure 1A. In this series, the probability of occurrence of SC was 1.6%

TABLE 4.—Characteristics of patients with SC[a]

Characteristic	
Total No. of patients	33 (3.0%)
Sex ratio	2.0
Age at diagnosis of HD	
Mean (yr)	42 (14 SD)
40–49 yr	6
≥50 yr	11
Age at diagnosis of SC	
Mean (yr)	49 (14 SD)
40–49 yr	6
≥50 yr	15
Mean interval from diagnosis of HD to SC (mo)	81 (52 SD)
CS	
I	12
II	21
Treatment group	
Mantle-field RT	2
Mantle-field RT + VLB	4
Mantle-field + PA ± spleen RT	7
Mantle-field + PA ± spleen RT + VLB	3
Mantle-field + PA ± spleen RT + VLB/NAT	6
Total lymphoid RT	3
MOPP × 3 + involved-field RT	0
MOPP × 3 + mantle-field + PA RT	0
MOPP × 6 + mantle-field RT	2
MOPP × 6 + involved-field RT	4
MOPP × 6 + mantle-field + PA RT	2

[a] Unless otherwise specified, values = No. of patients.

TABLE 5.—Outcome in 33 SC patients in complete remission from HD, by tumor type, treatment, sex, and age

Tumor type	Initial treatment	Sex	Age at onset of HD (yr)	Delay from HD to SC (mo)	Status on July 1, 1986
ANLL ($n=5$)	Mantle-field RT	M	56	92	Deceased
	Total lymphoid RT	M	55	45	Deceased
	MOPP×6+involved-field RT	M	40	10	Deceased
	MOPP×6+involved-field RT	M	69	18	Deceased
	MOPP×6+involved-field RT	F	30	53	Deceased
NHL ($n=5$)	Mantle-field RT+VLB	M	51	184	Deceased
	Mantle-field+PA RT+VLB	F	44	114	Deceased
	Mantle-field+PA RT+VLB/NAT	M	39	151	Alive
	MOPP×6+involved-field RT	F	57	46	Deceased
	MOPP×6+mantle-field+PA RT	M	25	56	Deceased
ST in irradiated area ($n=16$)					
Breast carcinoma	Mantle-field RT+VLB	F	32	155	Alive
Breast carcinoma	Mantle-field RT+VLB	F	44	188	Alive
Breast carcinoma	Mantle-field+PA RT	F	31	18	Deceased
Breast carcinoma	Mantle-field+PA RT	F	42	38	Alive
Bronchus carcinoma	Mantle-field+PA RT+VLB/NAT	M	38	108	Deceased
Bronchus carcinoma	Mantle-field+PA RT	M	57	108	Deceased
Gastric carcinoma	Mantle-field+PA RT+VLB/NAT	M	56	115	Deceased
Gastric carcinoma	Mantle-field+PA RT+VLB/NAT	M	19	150	Deceased
Sigmoid carcinoma	Mantle-field+PA RT	F	29	47	Alive
Sigmoid carcinoma	Mantle-field+PA RT+VLB	M	55	72	Deceased
Left kidney carcinoma	Mantle-field+PA+spleen RT+VLB/NAT	M	29	126	Deceased
Pharyngeal carcinoma	Mantle-field+PA RT	M	34	36	Deceased
Ovarian carcinoma	Total lymphoid RT	F	32	30	Alive
Bronchus carcinoma	Total lymphoid RT	M	69	41	Deceased
Bronchus carcinoma	MOPP×6+mantle-field RT	M	52	46	Deceased
Gastric carcinoma	MOPP×6+mantle-field+PA RT	M	42	19	Deceased
ST in nonirradiated area ($n=7$)					
Gastric carcinoma	Mantle-field RT+VLB	M	29	125	Deceased
Testicular carcinoma	Mantle-field RT	M	25	33	Alive
Right kidney carcinoma	Mantle-field+PA RT	M	63	110	Deceased
Melanoma	Mantle-field+PA RT+VLB	M	26	158	Alive
Anal carcinoma	Mantle-field+PA RT+VLB/NAT	F	49	96	Alive
Cerebral carcinoma	Mantle-field+PA RT	M	35	37	Deceased
Melanoma	MOPP×6+involved-field RT	F	27	68	Deceased

at 5 years after diagnosis of HD, 4.1% after 10 years, and 7.6% after 15 years. The cumulative proportions of SC, ANLL, NHL, and ST are shown in table 7; figure 1B shows the risk of ANLL and NHL; and figures 1C and 1D show the risk of ST. We observed similar probabilities for occurrence of ANLL and NHL with time for the first 10 years; thereafter, we observed an increase only in the probability of NHL (fig. 1B). Nevertheless, after 10 years, the overall probability of occurrence for ANLL was 0.7%; that for NHL was 1.2% after 15 years. In patients treated by combination MOPP plus RT, the 5-year cumulative proportion for ANLL was 0.8% and that for NHL was 0.6%. The cumulative proportion of all ST by treatment group is shown in figure 1C. There was a significant difference between the results in the group of patients treated by extended-field RT and in those treated by regional RT ($P=.01$). This difference was mainly due to an increased risk of ST in an irradiated area after extended-field RT (fig. 1D; $P=.009$).

Cox Model Analysis

The RR of SC was estimated for extended-field RT relative to regional RT; for chemotherapy with VLB or VLB/NAT relative to no chemotherapy; and for MOPP therapy relative to no MOPP therapy. All RR estimates were adjusted for the potential confounding effects of sex, CS, and age at diagnosis (0–39 or 40–70 yr). Table 8 gives the various RR for a model allowing all of the variables simultaneously. Three separate analyses were performed taking into account all SC, all ST, or ST localized in an irradiated area. No analysis could be performed for ANLL or NHL because of the small number of patients with these types of SC. Regardless of the type of SC considered, sex and CS had no significant influence on risk of occurrence of SC. When all SC were considered, the major risk factor was age (RR=4.66; $P<.001$), and extended-field RT had a slight but significant effect (RR=2.36; $P=.02$), while other types of treatment had no effect on risk. The same conclusions were made when all ST were considered separately but with a reduced effect of age (RR=3.09; $P=.005$) and an increased influence of extended-field RT (RR=3.90; $P=.008$). When only ST localized in an irradiated area were considered in the analysis, the effects of age (RR=4.41; $P=.003$) and extended-field RT (RR=7.20; $P=.008$) were greater.

Table 6.—General population comparisons: risk of SC[a]

Tumor type	Treatment	No. of years after treatment	No. of patients	Person-years at risk	No. of SC		RR (O/E)	95% confidence limits	*P* value
					Observed	Expected			
ANLL	RT ± single-agent CT	0–4	668	3,031	1	0.101	9.90	0.25–90	NS
		5–9	488	1,819	1	0.074	13	0.34–75	NS
		≥10	244	954	0	0.043	—	0–86	NS
		All	668	5,804	2	0.217	9.22	1.12–33	.002
	RT + MOPP	0–4	416	1,847	3	0.076	39	8.14–115	<.001
		5–9	232	338	0	0.015	—	0–246	NS
		All	416	2,185	3	0.090	33	6.87–97	<.001
	Both groups	All	1,084	7,989	5	0.307	16	5.29–38	<.001
NHL	RT ± single-agent CT	0–4	668	3,031	0	0.085	—	0–43	NS
		5–9	488	1,819	1	0.062	16	0.41–90	NS
		≥10	244	954	2	0.038	53	6.37–190	<.001
		All	668	5,804	3	0.184	16	3.36–48	<.001
	RT + MOPP	0–4	416	1,847	2	0.064	31	3.78–113	<.001
		5–9	232	338	0	0.011	—	0–335	NS
		All	416	2,185	2	0.074	27	3.27–98	<.001
	Both groups	All	1,084	7,989	5	0.258	19	6.29–45	<.001
ST in irradiated area	Regional or involved-field RT ± CT	0–4	534	2,401	1	3.501	0.29	0.01–1.59	NS
		5–9	353	1,062	0	1.530	—	0–2.41	NS
		≥10	134	794	2	1.631	1.23	0.15–4.43	NS
		All	534	4,257	3	6.229	0.45	0.09–1.32	NS
	Extended-field RT ± CT	0–4	550	2,477	7	3.497	2.00	0.80–4.12	NS
		5–9	367	1,095	4	2.166	1.85	0.50–4.73	NS
		≥10	110	160	2	0.370	5.41	0.65–20	NS
		All	550	3,732	13	6.007	2.16	1.15–3.70	.004
	Both groups	All	1,084	7,989	16	12.636	1.27	0.72–2.06	NS
ST in nonirradiated area	Regional or involved-field RT ± CT	0–4	534	2,401	1	3.501	0.29	0.01–1.59	NS
		5–9	353	1,062	1	1.530	0.65	0.02–3.64	NS
		≥10	134	794	1	1.631	0.61	0.02–3.42	NS
		All	534	4,257	3	6.229	0.45	0.09–1.32	NS
	Extended-field RT ± CT	0–4	550	2,477	1	3.497	0.29	0.01–1.59	NS
		5–9	367	1,095	2	2.166	0.92	0.11–3.34	NS
		≥10	110	160	1	0.370	2.70	0.07–15	NS
		All	550	3,732	4	6.007	0.67	0.18–1.71	NS
	Both groups	All	1,084	7,989	7	12.636	0.55	0.21–1.14	NS

[a] CT = chemotherapy; NS = not significant.

Impact of Second Cancer on Overall Survival

What was the consequence of occurrence of SC on overall survival of patients potentially cured from HD? In our series, 85% of the whole population were still alive 15 years after initial therapy. Most of the deaths were treatment related in older patients. After correction for increased mortality from SC, the proportion of surviving patients was 90% at 15 years (fig. 2).

DISCUSSION

The present study focuses on estimates of SC risk with time in relation to initial therapy in patients in first complete remission from early stages of HD. Quantitative estimate of risk of SC in HD patients is of great interest because survival improved dramatically with the use of aggressive multimodal therapy. Moreover, a greater proportion of patients with either early or advanced stages can now be cured with initial therapy and will never develop a disease recurrence (*23,24*). Thus, studies of series in which only initial therapy was administered may provide very informative data.

The present series can be considered as particularly useful for this research because it consisted of patients enrolled in successive clinical trials who had prospective follow-up. Although this series included only patients with early stages of HD, our conclusions can reasonably be extended to a majority of patients in first complete remission from HD, because initial therapy is determined by initial characteristics (*23*). Despite the fact that patients were selected, the use of the entire cohort with follow-up censored at the time of the relapse does not change the results, since 92% of the relapses occurred within the first 5 years after the initial treatment ended.

There are very few published series in which the results are focused on risk of SC in patients in first complete remission from HD (*25–32*). Nevertheless, a review of the literature provides some interesting data. After RT alone (regional or extended-field), the risk of secondary ANLL is very low. Curtis et al. (*30*) reported an RR of 2.5 ($P < .01$)

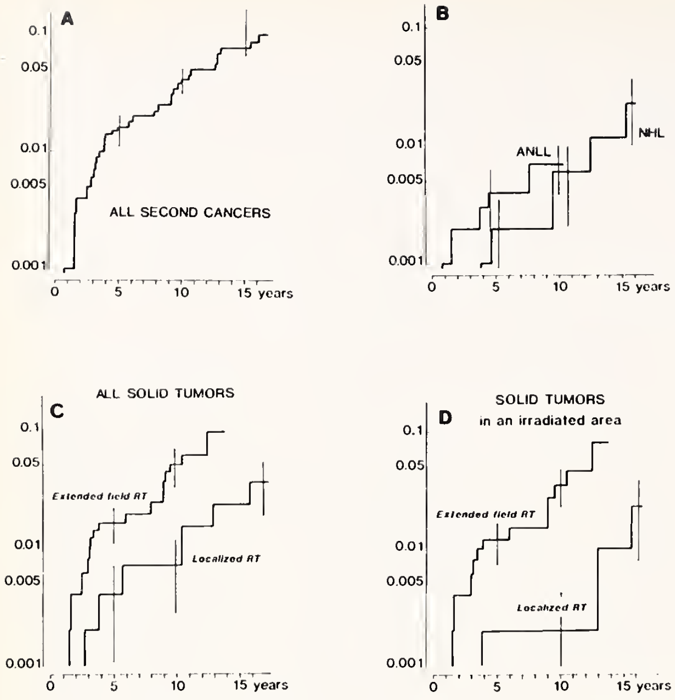


FIGURE 1.—A) Cumulative proportion of SC (±1 SE). B) Cumulative proportion of ANLL and NHL (±1 SE). C) Cumulative proportion of ST by treatment group (±1 SE) (log-rank test, $P=.01$). D) Cumulative proportion of ST in an irradiated area by treatment group (±1 SE) (log-rank test, $P=.009$).

after RT for various primary tumors; RR increased with time from initial treatment. Only one of the major published studies has reported a case of ANLL occurring after RT alone for HD (*26*). In addition, 2 cases are reported in the present study. After initial combined modality treatment, the cumulative proportion of ANLL ranges from 0% to 17.4% at 10 years (*33*). Heterogeneity in results appears to depend on the type of combination chemotherapy. Use of MOPP, combination MOPP plus RT, or combination alkylating agents plus RT induced higher risks than other modalities such as ABVD or combination ABVD plus RT (*32*). Recently, van Putten and van der Velden (*34*) reported from a case-control study that vincristine, NAT, mechlorethamine, extensive RT, and splenectomy were associated with ANLL risk. This study confirms the results published by Boivin et al. (*3*) and supports the finding that MOPP may be one of the major regimens that induces leukemia.

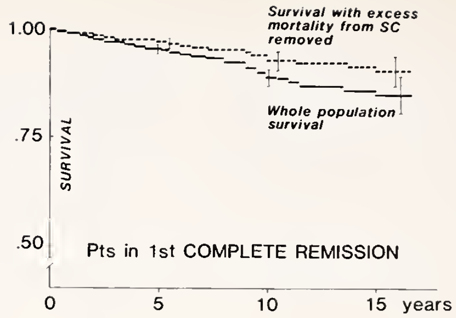


FIGURE 2.—Life-table analysis showing impact of SC on whole population survival (±1 SE).

The risk of secondary NHL was not analyzed until 1979, when Krikorian et al. (*10*) reported 6 cases of NHL following primary HD. Two of these patients were in first complete remission after combined modality treatment with RT plus MOPP. Furthermore, these investigators reported similar 10-year actuarial risks of NHL and ANLL in the whole study group. Boivin and Hutchison (*11*) reported risks of NHL similar to those reported in the present study. Nevertheless, in patients who developed secondary NHL, the delay between primary HD diagnosis and that of SC seems to differ depending on whether they were given combined modality treatments or RT alone (*26,31,32,35–38*). In many of the patients who received RT alone, the delay was >10 years. Thus, in patients in first complete remission, the major difference between the occurrence of NHL and ANLL appeared to be a greater RR for NHL during a period ≥10 years after RT.

When ANLL and NHL are considered overall, age seems to be of particular importance; this is in accordance with the fact that NHL and ANLL are more common in older people, as shown by incidence data relating to the general population. In addition, incidence rates of ANLL and NHL in the general adult population are similar, with a considerable increase at >40 years of age. Nevertheless, specific incidence rates never rise above 20–30/100,000 inhabitants per year (*39*). Consequently, a cumulative proportion of 1% of patients developing ANLL or NHL 10 years after treatment for HD suggests a potential carcinogenic effect of specific therapy.

No SC has been observed in the EORTC series of patients treated by 2-year adjuvant chemotherapy with NAT, in which the total dose is much greater than that administered after six cycles of MOPP. This suggests that NAT may not be the most carcinogenic component in the

TABLE 7.—Cumulative proportions of SC at 5, 10, and 15 yr by treatment category

Tumor type	Mode of therapy	% SC (SE) at yr— 5	10	15	Adjusted P value
All cancers	All patients	1.6 (0.4)	4.1 (0.9)	7.6 (1.7)	
ANLL	All patients	0.4 (0.2)	0.7 (0.3)	—	
NHL	All patients	0.2 (0.2)	0.6 (0.4)	1.2 (0.8)	
All ST	Regional or involved-field RT±CT	0.4 (0.3)	0.7 (0.4)	2.3 (1.2)	.01
	Extended-field RT±CT	1.6 (0.6)	5.0 (1.5)	13.5 (5.4)	
ST in irradiated area	Regional or involved-field RT±CT	0.2 (0.2)	0.2 (0.2)	1.0 (0.9)	.009
	Extended-field RT±CT	1.2 (0.5)	3.5 (1.3)	8.2 (3.8)	

TABLE 8.—Cox model: RR for model allowing all variables simultaneously

Variable	All cancers		All ST		ST in irradiated area	
	RR	*P* value	RR	*P* value	RR	*P* value
Age (40–70/0–39 yr)	4.66	<.001	3.09	.005	4.41	.003
Sex (M/F)	1.39	NS	1.32	NS	1.30	NS
CS of HD (II/I)	1.68	NS	1.17	NS	1.15	NS
Treatment						
Extended-field RT/regional RT	2.36	.02	3.90	.008	7.20	.008
VLB or VLB+NAT/no CT	1.24	NS	1.34	NS	1.55	NS
MOPP/no MOPP	1.26	NS	0.69	NS	0.82	NS
Global chi-square	38.38		22.09		24.89	
P value	<.001		.009		.003	

MOPP regimen (*14,15*). We must determine which is the more carcinogenic: alkylating agents or the combination of alkylating agents and RT (*33*). However, the potential additive effect of RT used with alkylating agents has not yet been precisely analyzed.

In considering ST following treatment for HD, many studies have emphasized that RT is the major carcinogenic agent. This knowledge derives from epidemiologic studies in which it has been shown that, with the exception of leukemia, the latency period for cancer occurrence after low-dose radiation exposure is usually >15–20 years (*40*). This finding is also true for NHL (*41*). Cumulative incidence analyses of occurrence of secondary ST after high-dose RT only provide information about cumulative risk with time; they provide no information on risk relative to the general population with no previous malignant disease. Nevertheless, these kinds of analyses provide information complementary to accumulation of person-year studies. In the present study, we did not observe any increase in risk of secondary ST with time, regardless of whether patients received regional or extended-field RT, which is contradictory to the findings in some other series (*3,25,33,42,43*). At 10 years, cumulative proportions of ST range from 0% after chemotherapy alone (*26,32,33*) or RT alone (*31,44,45*) to ≥10% for various treatments, mainly combined modality treatment (*26,32,46*). Unfortunately, none of these studies provided separate analysis by type of treatment; RT was given as regional or extended-field RT. In the present study, the cumulative proportion of SC, especially ST in an irradiated area, was greater in the group that received extended-field RT than in the group that received regional RT, mainly due to second primary tumors localized in the gastrointestinal tract. Moreover, no additive effect of chemotherapy or MOPP therapy to that of RT was found. Among patients who developed SC in a nonirradiated area, 2 had cutaneous malignant melanomas. None of these patients had a known family history of melanoma or a dysplastic nevus syndrome, unlike the patients studied by Tucker et al. (*47*).

Finally, occurrence of SC had a small impact on overall mortality, which was greater than that reported by Rubin et al. (*33*). Furthermore, this impact could increase with time if the efforts to better adapt the treatment to initial characteristics of the disease become increasingly effective.

CONCLUSIONS

The data presented in this report provide original information because none of the patients included in the study was given additional treatment after initial therapy. Therefore, the estimated risks of secondary ANLL, NHL, and ST must be considered as estimations of potential severe late effects related to initial therapy. Although the risk of SC observed is lower than that generally reported by other investigators, its influence on the overall survival rate of patients with high probability of cure is far from negligible. The effect of the type of therapy on the risk of secondary NHL remains unclear. Additional studies are necessary to determine whether RT, combination chemotherapy, or age have a significant additional impact on occurrence of NHL. Finally, the risk of occurrence of secondary ST does not appear to be significantly increased relative to incidence rates in the general population, although a slight tendency toward increase with time was observed for ST in an irradiated area after extended-field RT. This finding and the absence of increased risk of secondary ANLL after RT should encourage physicians to avoid combination chemotherapy when possible in the treatment of early stages of HD. Nevertheless, the data suggest that patients treated for HD should have careful follow-up for at least 15–20 years, in order to better estimate the risks of SC.

REFERENCES

(*1*) Editorial: Second malignancies in lymphoma patients. Lancet 2:1163–1164, 1985.

(*2*) Coleman CN: Editorial: Secondary malignancies after treatment for Hodgkin's disease: An evolving picture. J Clin Oncol 4:821–824, 1986.

(*3*) Boivin JF, Hutchison GB, Lyden M, et al: Second primary cancers following treatment of Hodgkin's disease. JNCI 72:233–241, 1984.

(*4*) Hayat M: A randomized study of irradiation and vinblastine in clinical stages I and II of Hodgkin's disease. Preliminary results. Eur J Cancer 8:353–362, 1972.

(*5*) van der Werf-Messing B: Morbus Hodgkin's disease, stages I and II: Trial of the European Organization for Research on Treatment of Cancer. Natl Cancer Inst Monogr 36:381–386, 1973.

(*6*) Tubiana M, Henry-Amar M, Hayat M, et al: Long-term results of the EORTC randomized study of irradiation and vinblastine in clinical stages I and II of Hodgkin's disease. Eur J Cancer 15:645–657, 1979.

(*7*) Tubiana M, Hayat M, Henry-Amar M, et al: Five-year results of the EORTC randomized study of splenectomy and spleen irradiation in clinical stages I and II of Hodgkin's disease. Eur J Cancer 17:355–363, 1981.

(*8*) Tubiana M, Henry-Amar M, Hayat M, et al: The EORTC treatment of early stages of Hodgkin's disease: The role of radiotherapy. Int J Radiat Oncol Biol Phys 10:197–210, 1984.

(*9*) Zittoun R, Audebert A, Hoerni B, et al: Extended versus involved fields irradiation combined with MOPP chemotherapy in early clinical stages of Hodgkin's disease. J Clin Oncol 3:207–214, 1985.

(*10*) Krikorian JG, Burke JS, Rosenberg SA, et al: Occurrence of non-Hodgkin's lymphoma after therapy for Hodgkin's disease. N Engl J Med 300:452–458, 1979.

(*11*) Boivin JF, Hutchison GB: Leukemia and other cancers after radiotherapy and chemotherapy for Hodgkin's disease. JNCI 67:751–760, 1981.

(*12*) Jacquillat C, Khayat D, Desprez-Curely JP, et al:

Non-Hodgkin's lymphoma occurring after Hodgkin's disease. Cancer 53:459–462, 1984.

(*13*) Fisher RI: Editorial: Non-Hodgkin's lymphomas after treatment of Hodgkin's disease. Arch Intern Med 143:427, 1983.

(*14*) Henry-Amar M: Second cancers after treatment in two successive cohorts of patients with Hodgkin's disease. *In* Malignant Lymphomas and Hodgkin's Disease: Experimental and Therapeutic Advances (Cavalli F, Bonadonna G, Rozencweig M, eds). Boston: Martinus Nijhoff, 1985, pp 417–428.

(*15*) Carde P, Henry-Amar M, Tubiana M, et al: No increased incidence of second leukemias in patients treated with vinblastine alone or associated with procarbazine following radiotherapy in two successive EORTC controlled trials (1964–1976) in clinical stages I–II Hodgkin's disease. Proc ASCO 4:212, 1985.

(*16*) Schaffer P, Lavillaureix J (eds): Le Cancer dans le Bas-Rhin: Incidence des Nouveaux Cas de 1975 à 1977. Paris: Economica, 1981.

(*17*) Netherlands Central Bureau of Statistics: Cancer morbidity and mortality 1975–1976. The Hague: Staatsuitgeverij, 1979.

(*18*) Schoenberg BS, Myers MH: Statistical methods for studying multiple primary neoplasms. Cancer 40:1892–1898, 1977.

(*19*) Mantel N: Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 50:163–170, 1966.

(*20*) Makuch R, Simon R: Recommendations for the analysis of the effect of treatment on the development of second malignancies. Cancer 44:250–253, 1979.

(*21*) Cox DR: Regression model and life-tables. J R Stat Soc 33:187–220, 1972.

(*22*) Breslow N: Statistical methods for censored survival data. Environ Health Perspect 32:181–192, 1979.

(*23*) Kaplan HS: Hodgkin's Disease, 2nd ed. Cambridge, MA: Harvard Univ Press, 1980.

(*24*) Bonadonna G: Chemotherapy of malignant lymphomas. Semin Oncol 12(suppl 6):1–14, 1985.

(*25*) Nelson DF, Cooper S, Weston MG, et al: Second malignant neoplasms in patients treated for Hodgkin's disease with radiotherapy and chemotherapy. Cancer 48:2386–2393, 1981.

(*26*) Coleman CN, Kaplan HS, Cox R, et al: Leukemias, non-Hodgkin's lymphomas and solid tumors in patients treated for Hodgkin's disease. Cancer Surv 1:733–744, 1982.

(*27*) Coleman CN: Secondary neoplasms in patients treated for cancer: Etiology and perspective. Radiat Res 92:188–200, 1982.

(*28*) Glicksman A, Pajak TF, Gottlieb A, et al: Second malignant neoplasms in patients successfully treated for Hodgkin's disease: A Cancer and Leukemia Group B Study. Cancer Treat Rep 66:1035–1044, 1982.

(*29*) Henry-Amar M: Second cancers after radiotherapy and chemotherapy for early stages of Hodgkin's disease. JNCI 71:911–916, 1983.

(*30*) Curtis RE, Hankey BF, Myers MH, et al: Risk of leukemia associated with the first course of cancer treatment: An analysis of the Surveillance, Epidemiology, and End Results Program experience. JNCI 72:531–544, 1984.

(*31*) Tester WJ, Kinsella TJ, Waller B, et al: Second malignant neoplasms complicating Hodgkin's disease: The National Cancer Institute experience. J Clin Oncol 2: 762–769, 1984.

(*32*) Valagussa P, Santoro A, Fossati-Bellani F, et al: Second acute leukemia and other malignancies following treatment for Hodgkin's disease. J Clin Oncol 4:830–837, 1986.

(*33*) Rubin P, Zagars G, Chuang C, et al: Hodgkin's disease: Is there a price for successful treatment? A 25-year experience. Int J Radiat Oncol Biol Phys 12:153–166, 1986.

(*34*) van Putten WLJ, van der Velden JW: Development of acute nonlymphocytic leukemia after treatment for Hodgkin's disease. Methodological aspects of a case control study of the International Cancer Patient Data Exchange System (ICPDS). Presented at the Seventh Meeting of the International Society for Clinical Biostatistics, Cardiff, Wales, Sept 15–19, 1986.

(*35*) D'Agostino RS: Non-Hodgkin's lymphoma after chemotherapy for Hodgkin's disease. N Engl J Med 301:1289, 1979.

(*36*) Kim H, Bedetti C, Boggs DR: The development of non-Hodgkin's lymphoma after therapy for Hodgkin's disease. Cancer 46:2596–2602, 1980.

(*37*) Schomberg PJ, Evans RG, Banks PM, et al: Second malignant lesions after therapy for Hodgkin's disease. Mayo Clin Proc 59:493–497, 1984.

(*38*) Koletsky AG, Bertino JR, Farber LR, et al: Second neoplasms in patients with Hodgkin's disease following combined modality therapy. The Yale experience. J Clin Oncol 4:311–317, 1986.

(*39*) Waterhouse J, Muir C, Shanmugaratman K, et al (eds): Cancer Incidence in Five Continents, vol IV. International Agency for Research on Cancer, scientific publication No. 42. Lyon, France: International Agency for Research on Cancer, 1982.

(*40*) Schull WJ: Atomic bomb survivors: Pattern of cancer risk. *In* Radiation Carcinogenesis: Epidemiology and Biological Significance (Boice JD Jr, Fraumeni JF Jr, eds). New York: Raven Press, 1984, pp 21–36.

(*41*) Finch SC: Leukemia and lymphoma in atomic bomb survivors. *In* Radiation Carcinogenesis: Epidemiology and Biological Significance (Boice JD Jr, Fraumeni JF Jr, eds). New York: Raven Press, 1984, pp 37–44.

(*42*) Greene MH, Wilson J: Second cancer following lymphatic and hematopoietic cancers in Connecticut, 1935–1982. Natl Cancer Inst Monogr 68:191–217, 1985.

(*43*) Storm HH, Prener A: Second cancer following lymphatic and hematopoietic cancers in Denmark, 1943–1980. Natl Cancer Inst Monogr 68:389–409, 1985.

(*44*) Baccarani M, Bosi A, Papa G: Second malignancy in patients treated for Hodgkin's disease. Cancer 46:1735–1740, 1980.

(*45*) Coltman CA, Dixon DO: Second malignancies complicating Hodgkin's disease: A Southwest Oncology Group 10-year follow-up. Cancer Treat Rep 66:1023–1033, 1982.

(*46*) Brody RS, Schottenfeld D, Reid A: Multiple primary cancer risk after therapy for Hodgkin's disease. Cancer 40:1917–1926, 1977.

(*47*) Tucker MA, Misfeldt D, Coleman N, et al: Cutaneous malignant melanoma after Hodgkin's disease. Ann Intern Med 102:37–41, 1985.

Discussion Summary

Anthony E. Howes[1],* and Mark E. Nesbit, Jr.[2]

Discussion following the first session began by questions on the usefulness of experimental models used in the study of normal tissue injury, particularly injury related to chemotherapy. It was generally agreed that there is a marked shortage of such systems, particularly with regard to the study of chronic or late effects; so far experiments tend not to be addressed to current clinical questions. The best studied model is that for the rodent kidney response to cisplatin and radiation. As both modalities are known to produce renal damage, it is not surprising that interactions can be demonstrated experimentally, as shown by several participants. Of particular clinical relevance was the observation that the extent of the interaction is critically dependent on the temporal spacing of radiation and drug; the greatest effect is seen when cisplatin is given several weeks after exposure of kidneys to x-rays. This important experimental observation should alert clinicians to look for similar interactions in their patients. Interestingly, no such interaction has been reported, perhaps because clinically defined tolerance levels may be lower than necessary, and additive effects may not be great enough to produce clinically observable injury. This possibility stresses the need for better means of detecting subclinical levels of effect, a theme which recurred several times throughout the discussion period. It was also strongly suggested that more laboratory work needs to be done with cisplatin and radiation with other normal tissues that are currently of most concern clinically, e.g., oropharyngeal and esophageal mucosa. Because of the lack of adequately developed techniques for studying the detailed response of normal tissues to chemotherapeutic agents, it is expected that most investigators doing laboratory research will focus on how drugs modify the response of tissues to single or fractionated doses of radiation.

In the area of clinical models of normal tissue response, data are even more scarce. Although important interactions between radiation and drugs in the production of serious late effects (such as leukoencephalopathy in leukemia patients) have been clearly documented by careful retrospective analysis of clinical material, major difficulties prevent the collection of such data in a prospective manner. This is mainly because the prediction of many adverse effects is difficult and they may occur after many years, so that a very large amount of baseline and follow-up information would be required. Support of such studies would require close cooperation of several treatment centers or multicenter groups plus a commitment for expensive long-term funding that would not lend itself readily to the normal means of evaluation by peer review. Dr. Nesbit pointed out that 10 years ago the Children's Cancer Study Group set up a Late Effects Study Committee whose function is to determine which studies should be performed on a long-term basis following initial assessment of disease status. This approach has usually worked well, although there are occasional problems of compliance on the part of follow-up examiners. It was generally believed that such an approach would be of value for adult patients, particularly those participating in combined modality protocols. Dr. Wasserman reported that representatives of all cooperative groups met recently in Baltimore to establish guidelines for collecting data concerning late normal tissue effects following radiotherapy. The recommendations from this meeting will be published shortly and, hopefully, will be acceptable for both radiation and combined modality research. With regard to the need for assays of radiation or drug injury at subclinical levels, Dr. Rubin described the exciting prospect that soon we may be able to monitor tissue injury by measuring serum levels of certain biochemical compounds. This approach is currently being investigated with surfactant as a marker for lung injury and myelin basic protein for damage to the spinal cord.

---

[1] Division of Cancer Biology, Joint Center for Radiation Therapy, Harvard Medical School, Boston, MA.

[2] Department of Pediatrics, University of Minnesota School of Medicine, Minneapolis.

* *Reprint requests:* Anthony E. Howes, M.D., Division of Cancer Biology, Joint Center for Radiation Therapy, Harvard Medical School, 50 Binney St., Boston, MA 02115.

Mechanisms of Interaction/Resistance—I

Keynote Address: Mechanisms of Interaction Between Radiation and Drugs With Potential for Improvements in Therapy[1]

Ian F. Tannock[2,3,*] **and Daniela Rotin**[1,3]

The proceedings of the Conference on Combined Modalities: Chemotherapy/Radiotherapy, which was held at Hilton Head Island, SC, in 1978 (*1*), show that the combined effect of radiation with any of several drugs is not easily predictable from the effect of either agent used alone. While some combinations hold promise of therapeutic benefit, the effect of combined treatment often depends critically on the doses of radiation and drugs, the schedule, and the end point studied. As Dr. Charles G. Moertel concluded in summarizing one of the sessions:

> "Based on the results of various individual studies, I could conclude that it is most ideal to administer the nitrosourea 15 hours before irradiation, 2 hours before irradiation, simultaneously with irradiation, or 6 hours after irradiation. While we will continue to cheer our radiation biology colleagues on from the sidelines, I am afraid we are not yet at the stage where we can comfortably incorporate their results into our clinical practice."

This statement applies, with minor modification, to the results of studies using radiation with several drugs. Limited ability to apply the results of many experimental studies in clinical medicine may be due in part to their phenomenological nature. Most investigators have characterized the results of combined treatment in one or more model systems, but there have been relatively few attempts to study mechanisms that might lead to improved therapeutic outcome. It is our belief that results obtained in experimental systems will become applicable to humans only by the study of mechanisms and the design of combined modality experiments to test or refute a hypothesis based on known biologic properties of tumor and normal tissue.

MECHANISMS FOR THERAPEUTIC BENEFIT FROM COMBINED MODALITIES

Combined modality treatment has most often employed radiation therapy to sites of bulky disease, usually the primary tumor, and chemotherapy has been directed against known or suspected metastases. If the radiation field is small, the different dose-limiting toxic effects (e.g., local tissue damage from radiation and myelosuppression from chemotherapy) may allow combined treatment at almost full doses of each agent. This treatment plan has improved therapeutic outcome for several human malignancies including lymphomas and pediatric tumors, although the inability of drugs to eradicate even micrometastases of most solid tumors in adults currently limits the value of this approach. When therapeutic benefit occurs, it does not necessarily imply any interaction between the two agents.

Use of combined modality treatment to improve therapeutic effects against a primary tumor requires some form of interaction or cooperation between radiation and drugs so that there is a greater increase in damage to the tumor cells than to the normal tissues in the radiation field. Therapeutic benefit is not dependent on synergistic interaction of the two modalities; indeed, interactions studied in a single population of cells have little relevance to therapy unless they are used to elucidate mechanisms, because even interactions that are antagonistic or subadditive might be therapeutically useful if the degree of antagonism were greater for normal tissue than for tumor.

Mechanisms by which drugs can interact with radiation to cause differential effects against tumor and normal tissue must depend on differences in the properties of these two types of tissue. Compilation of a list of such properties (table 1), therefore, provides a starting point for investigation of mechanisms that may be exploitable through the use of combined modality treatment.

Properties listed in table 1 are not exhaustive. Some mechanisms proposed by other investigators have been excluded. For example, use of one agent to induce synchrony that is then exploited by the other agent seems implausible in view of the wide heterogeneity in both tumors and normal tissues of such factors as cell-cycle properties and drug distribution (*2*). Other potential mechanisms might be added. Thus, if mechanisms of DNA repair differ between some tumors and normal tissues, drugs might be used to selectively inhibit DNA repair in these tumors.

Repopulation during fractionated radiotherapy may limit the ability of radiation therapy to control tumors that contain rapidly proliferating cells. Use of drugs to inhibit repopulation seems therefore to be exploitable; many human tumors, however, contain cells that proliferate at a rate similar to or slower than the rate in normal tissues included in the radiation field. Thus, even if this mechanism is exploitable, one should not expect therapeutic benefit in all clinical scenarios.

ABBREVIATIONS: CHO cells = Chinese hamster ovary cells; pH_i = intracellular pH; pH_e = extracellular pH; DIDS = 4,4′-diisothiocyanostilbene-2,2-disulfonic acid.

[1] Supported by Public Health Service grant CA-36913 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and by a grant from the National Cancer Institute of Canada. D. Rotin is the recipient of a National Cancer Institute of Canada Research Studentship.

[2] Department of Medicine, Ontario Cancer Institute, Toronto, Canada.

[3] Division of Physics, Ontario Cancer Institute, and Department of Medical Biophysics, University of Toronto, Canada.

[*] *Reprint requests:* Ian F. Tannock, M.D., Department of Medicine, Ontario Cancer Institute, 500 Sherbourne St., Toronto, Ontario M4X 1K9, Canada.

TABLE 1.—Differential properties of tumors and normal tissues with potential for therapeutic exploitation by combined modality treatment

Property	Potential mechanism for increased therapeutic effect through use of drugs
Genetic instability of tumors	Elimination of radioresistant clones by drugs, favoring tumor response, although drugs that stimulate mutation or gene amplification might induce radiation resistance
Rate of cell proliferation	Inhibition of tumor repopulation during fractionated radiation therapy
Cell-cycle effects	Prevention of redistribution to resistant phases of cycle, favoring tumor response
Hypoxia in tumors	Sensitization of hypoxic cells Selective toxicity for hypoxic cells Improvement of oxygen distribution
Lower pH in tumors	Sensitization of acidic cells to radiation pH-dependent cell killing
Cellular lysis after treatment	Stimulation of cumulative cell death, favoring tumor response More rapid shrinkage of tumors, influencing drug access and response or allowing larger radiation doses to smaller fields

IMPORTANCE OF HETEROGENEITY

Tumors have been shown to be heterogeneous with respect to almost any biologic property that can be characterized (*3*), including those listed in table 1. Processes that may contribute to heterogeneity in tumors are summarized in table 2. Each of these processes may lead to tumor subpopulations with different responses to therapeutic agents, and if response to radiation and some drugs is independent, different treatment modalities directed at different subpopulations may offer potential for improved therapeutic effects.

Heterogeneity in the genetic properties of malignant cells has been demonstrated most frequently through study of drug resistance or metastatic phenotypes. If cells are exposed continuously to an anticancer drug, stable and heritable drug resistance is usually achieved. Such resistance has been shown to occur through mechanisms of point mutation and/or stable amplification of genes (*4,5*). Examples are mutant cells deficient in the transport of methotrexate or cells amplified for the dihydrofolate reductase gene, which encodes the target enzyme for methotrexate. Although stable drug-resistant cells have been demonstrated in some tumors, the selection pressure applied in these experiments is usually much greater than in a human cancer treated with intermittent chemotherapy. Few experiments have attempted to simulate conditions under which drug resistance may develop clinically, but one example is provided by the experiments of McMillan et al. (*6*), who studied the development of resistance to several drugs during repeated treatment and passage of an experimental tumor. Drug resistance was found to develop gradually; various levels of resistance were observed among the population of tumor cells; and the phenotype could not be explained by selection from pre-existing drug-resistant populations. Varying levels of amplification or expression of genes could explain some of these results.

Studies of the metastatic phenotype suggest that metastatic variants may be produced and lost at a high rate, so that a state of dynamic heterogeneity may exist among malignant cells (*7*). The acquisition and loss of resistance to some drugs may occur in an analogous way (*8*), and a probable mechanism is the transient amplification of genes.

The possibility that environmental factors in tumors may stimulate amplification or expression of genes has been suggested by recent experiments. Wilson et al. (*9*) have demonstrated that hypoxia may stimulate production of specific proteins (oxygen-regulated proteins) in cultured cells. Rice et al. (*10*) have shown that transient exposure of cells to hypoxia stimulated the development of resistance to methotrexate through amplification of the dihydrofolate reductase gene. The probable mechanism relates to inhibition of DNA synthesis by hypoxia followed by subsequent over-replication of DNA and nonspecific amplification of genes. This process may also be stimulated by drugs that inhibit S-phase progression (*5*) and perhaps also by the

TABLE 2.—Processes with potential for producing heterogeneity in tumors

Process	Possible effect on therapeutic response
Point mutation	Generation of clones with different responsiveness to radiation and drugs
Changes in gene expression (e.g., amplification, DNA methylation)	Generation of resistance to therapy—may or may not be stable
Phenotypic changes in a clone (e.g., differentiation)	Varying ability of cells to regenerate after treatment
Heterogeneity of metabolite concentration resulting from deficient vasculature	Such factors as hypoxia and low pH, which may influence therapeutic response Variability in cell cycle parameters Variability in drug concentration
Interaction between cells with different properties	Prevention of ability to predict response on basis of studies of individual subpopulations Horizontal transmission of radiation or drug resistance (e.g., "contact effect") Cumulative cell death

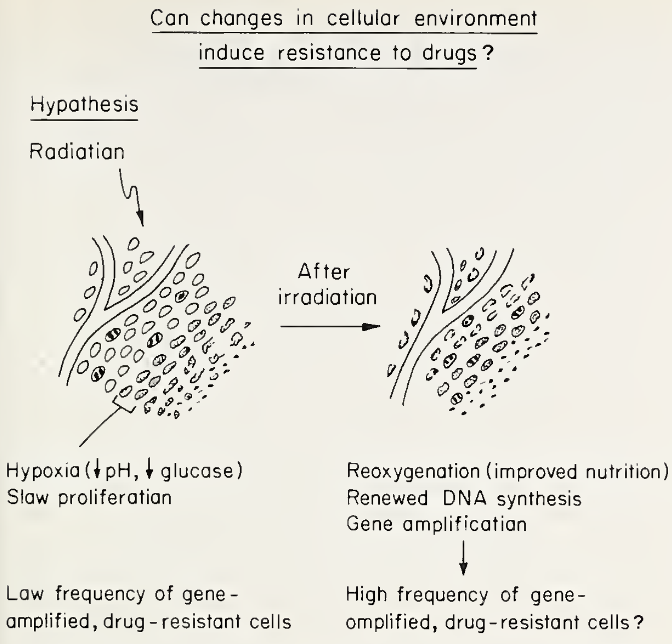

Can changes in cellular environment induce resistance to drugs?
Hypothesis
Radiation
After irradiation
Hypoxia (↓pH, ↓glucose)
Slow proliferation
Low frequency of gene-amplified, drug-resistant cells
Reoxygenation (improved nutrition)
Renewed DNA synthesis
Gene amplification
High frequency of gene-amplified, drug-resistant cells?

FIGURE 1.—Hypothetical mechanism whereby radiation treatment of solid tumor may promote sequence of reoxygenation, DNA synthesis in previously hypoxic cells, and amplification of genes that may convey drug resistance. Hypothesis is based on results of Rice et al. (*10*), which demonstrated that resistance to methotrexate of cells in culture could be stimulated after exposure to hypoxic conditions.

depletion of various nutrients that may limit cell proliferation in some regions of solid tumors. Resistance to many drugs, including the multidrug-resistance phenotype, may be caused by amplification of genes, and the same process may lead to radiation resistance through amplification of genes that encode enzymes involved in DNA repair. Thus, a potential problem in the treatment of solid tumors is that the reoxygenation of surviving cells after a given radiation dose or the improved nutrition of surviving cells after chemotherapy may promote over-replication of DNA, nonspecific amplification of genes, and resistance to therapy in some of these gene-amplified cells (fig. 1). If this hypothesis proves correct, it will have profound implications for the choice and sequence of radiation and drugs used in combination.

Whatever the mechanism by which subpopulations of cells with different properties are generated, their presence allows the potential for cooperative effects between different modalities. This is best illustrated by an idealized example of two subpopulations, each totally resistant to one modality but exquisitely sensitive to the other. Use of one modality could then achieve, at most, total cell kill of one subpopulation, with minimal effects on tumor growth, whereas use of both modalities could eradicate the whole population. In practice, properties that show heterogeneity in tumor populations (hypoxia, pH, and levels of resistance to radiation or drug therapy) have a continuous spectrum among the population, and agents with absolute specificity are not available. Nonetheless, the approach of using different modalities directed against cells at ends of the spectrum of any given biologic property holds promise for therapeutic gain.

HYPOXIC CELL AS TARGET OF DRUG TREATMENT

Since hypoxic cells are resistant to treatment with radiation, they are obvious targets for chemotherapy. Two mechanisms may contribute to hypoxia in tumors: limited diffusion of oxygen to cells distant from blood vessels and acute changes in blood flow. Chemotherapy using some clinically available drugs may be effective against acutely hypoxic cells if the half-life of the drug in the circulation exceeds the periodicity with which tumor blood vessels may open or close, although there will be no selective toxicity for such cells. In contrast, cells that are hypoxic because of diffusion limitations may be less likely to respond to conventional drugs because drug penetration may be limited and because the relatively slow rate of proliferation of these cells may convey drug resistance (*11–13*).

Two methods have been used to study the response of aerobic and hypoxic cells to anticancer drugs in experimental tumors; results are summarized in table 3. One method has used radiation treatment given under aerobic or hypoxic conditions to estimate the proportion of severely hypoxic cells in drug-treated and control tumors (*11–13*). An increase in the proportion of radiobiologically hypoxic cells after drug treatment then implies that the drug is selective for aerobic cells, whereas a decrease in the hypoxic fraction implies specificity for hypoxic cells. The technique gives no information about mechanisms. Doxorubicin is probably selective for aerobic cells because it has limited diffusion from tumor blood vessels, whereas misonidazole is thought to be selective for hypoxic cells because it is reduced to an active form under hypoxic conditions. Also, the method depends on assessment of the radiation response of a tumor, usually by growth delay or excision assay, under four different conditions: aerobic, hypoxic, drug treated, and control. Since each of these assessments is subject to uncertainty, the method can only detect quite large differences in the sensitivity of drugs for aerobic and hypoxic cells.

A technique that was devised initially to assess drug effects at different depths in spheroids has been applied to study drug effects at different distances from tumor blood vessels (*14,17*). The method is based on diffusion from

TABLE 3.—Relative activity of drugs for aerobic and hypoxic cells of experimental tumors

Drug	Method[a]	Result	Reference No.
Carmustine	A	Selective toxicity for aerobic cells	(*11*)
Cyclophosphamide	A	No selectivity (2 studies) Selective for aerobic cells (1 study)	(*11–13*)
Doxorubicin	A, B	Selective for aerobic cells	(*13,14*)
Mechlorethamine	A	Selective for aerobic cells	(*15*)
5-Fluorouracil	A	No selectivity	(*16*)

[a] A = assessment by subsequent treatment with radiation; B = assessment by Hoechst 33342 diffusion and fluorescence-activated cell sorting.

tumor blood vessels of the vital fluorescent stain, Hoechst 33342, which establishes a concentration gradient. The stain is injected into animals bearing control or drug-treated tumors. The tumors are then removed, and the cells are dissociated, separated by fluorescence-activated cell sorting on the basis of Hoechst fluorescence, and plated to assess cell survival. This method has confirmed that the toxicity of both radiation and doxorubicin decreases with increasing distance from a tumor blood vessel (*14*) or from the surface of spheroids (*17*). Surprisingly, the method has not shown a marked dependence of cellular location in spheroids on toxicity for many other drugs in clinical use; it does, however, indicate large differences in sensitivity (in either direction) of cells in intact spheroids, compared to single cells dissociated from them (*17*). The mechanisms underlying these differences in activity are unknown, but the existence of the differences casts doubt on the validity of using studies of drug response of single cells in culture to predict responsiveness in solid tumors. The Hoechst technique should prove useful in the study of drug effects at different distances from blood vessels in experimental tumors. Its major limitations are that the Hoechst stain may sometimes influence drug or radiation toxicity (*18*) and that vessels may open or close between the time of drug administration and the time that the Hoechst stain is injected to define patent blood vessels.

Some drugs are selectively toxic to hypoxic cells in tissue culture. These include nitroimidazoles such as misonidazole, glucose analogues, nitrobenzene and nitroacridine compounds, and bioreductive alkylating agents such as mitomycin or porfiromycin. Unfortunately, most of these agents either show no selectivity in vivo, perhaps because of poor penetration from blood vessels, or demonstrate selective activity only at doses that are toxic to the host. Several of these agents appear to have dose-limiting neurotoxicity. This effect might be expected for inhibitors of glycolysis such as 5-thio-D-glucose since the central nervous system is known to depend on this metabolic pathway. New drugs that appear to have toxicity for hypoxic cells in vivo at tolerated doses, such as RSU 1069 and SR 4233, are currently being tested in experimental systems (*19,20*). The development of these or other drugs with selective toxicity for hypoxic cells in tumors retains the potential for improving the therapeutic index through the use of combined treatment with radiation.

LOW pH IN TUMORS

Tumor cells that survive treatment with radiation may be susceptible to drugs through mechanisms that are related *indirectly* to the hypoxic state. One exploitable property of hypoxic cells may be their low pH. Mean pH in solid tumors has been found to be lower than in normal tissues (*21*), and since hypoxic cells depend on anaerobic glycolysis for a source of energy, the production of lactate probably leads to values of pH in hypoxic tumor regions that are considerably lower than the mean. Support for this hypothesis is provided by estimates of pH in spheroids; the pH in central hypoxic regions tends to be lower than in the periphery (*22*).

Our recent studies have been prompted by the apparent paradox that 1) hypoxia appears to be involved in processes leading to cell death in solid tumors (*23*) and 2) hypoxic cells maintained under otherwise normal conditions in tissue culture may survive for prolonged periods (*24*). We suspected that deprivation of nutrients other than oxygen or accumulation of toxic catabolites might interact with hypoxia to cause cell death. We and others have tested this hypothesis by varying the concentration of various metabolites in tissue culture or in the medium surrounding spheroids. One factor that may lead to necrosis in the center of spheroids appears to be the interaction of hypoxia with low levels of glucose (*25,26*). Another important factor that leads to the rapid death of cells in tissue culture is an interaction between hypoxia and acidic pH (*27*).

The interaction of hypoxia and low pH is toxic both for CHO cells and for human bladder carcinoma MGH-U1 cells. We have demonstrated that whereas cells are resistant to periods of hypoxia alone for 6 hours and to pH 6.0 under aerobic conditions, the combination of these factors may reduce cell survival to the range of 10^{-5} to 10^{-4} (fig. 2). Cell killing may be due partly to inhibition of energy metabolism since acid pH inhibits phosphofructokinase, the rate-limiting enzyme of glycolysis, and hypoxia inhibits respiration. However, additional mechanisms are inferred by the finding that exposure of cells to hypoxia in glucose-free medium, thereby eliminating both respiration and glycolysis, led to a more rapid fall in cellular ATP levels but to a lower rate of cell killing. Measurement of pH_i based on the distribution of the ^{14}C-labeled weak acid 5,5-dimethyl-2,4-oxazolidinedione suggests that hypoxia impairs the ability of ion exchangers in the cell membrane to protect pH_i when pH_e decreases (fig. 3).

Two classes of drugs might be selectively toxic at low pH: 1) drugs with pK_a values such that their activity or uptake increases at low pH or 2) drugs that interfere with the control of pH_i. One drug in the first category is the alkylating agent triethylenemelamine, and we have reported previously a study of the use of this drug with radiation to treat an experimental tumor (*29*). We were not able to demonstrate improved therapeutic effects, perhaps because the drug is unstable or has limited penetration from blood vessels. More recently, we have investigated agents that influence mechanisms that act to control pH_i.

At least two ion-exchange mechanisms are known to act to maintain the pH_i of mammalian cells (fig. 4): a sodium-proton exchanger, which is sensitive to amiloride, and a chloride-bicarbonate exchanger, which is sensitive to stilbene derivatives such as DIDS. Also, other types of drug, known as ionophores, act to transfer ions across cell membranes and may therefore disturb these control mechanisms. One ionophore, carbonylcyanide-3-chlorophenylhydrazone, has been shown to increase the response of murine cells to radiation under acidic conditions (*30*). Another ionophore, nigericin, selectively exchanges H^+ for K^+ ions and, under appropriate conditions, prevents maintenance of a pH gradient across the cell membrane.

We have studied the pH-dependent effects of nigericin, amiloride, and DIDS on CHO and MGH-U1 cells in tissue culture (*28*). Nigericin was ineffective above pH 6.5 but caused cell killing as pH was reduced below this value. Amiloride and DIDS alone were not toxic, but they showed major effects in increasing the pH-dependent cell killing of nigericin. Moreover, when the three drugs were used together, the range of pH_e over which toxicity was observed increased to 6.5–6.9 (fig. 5). Many tumors have been reported to have mean values of pH_e within this range

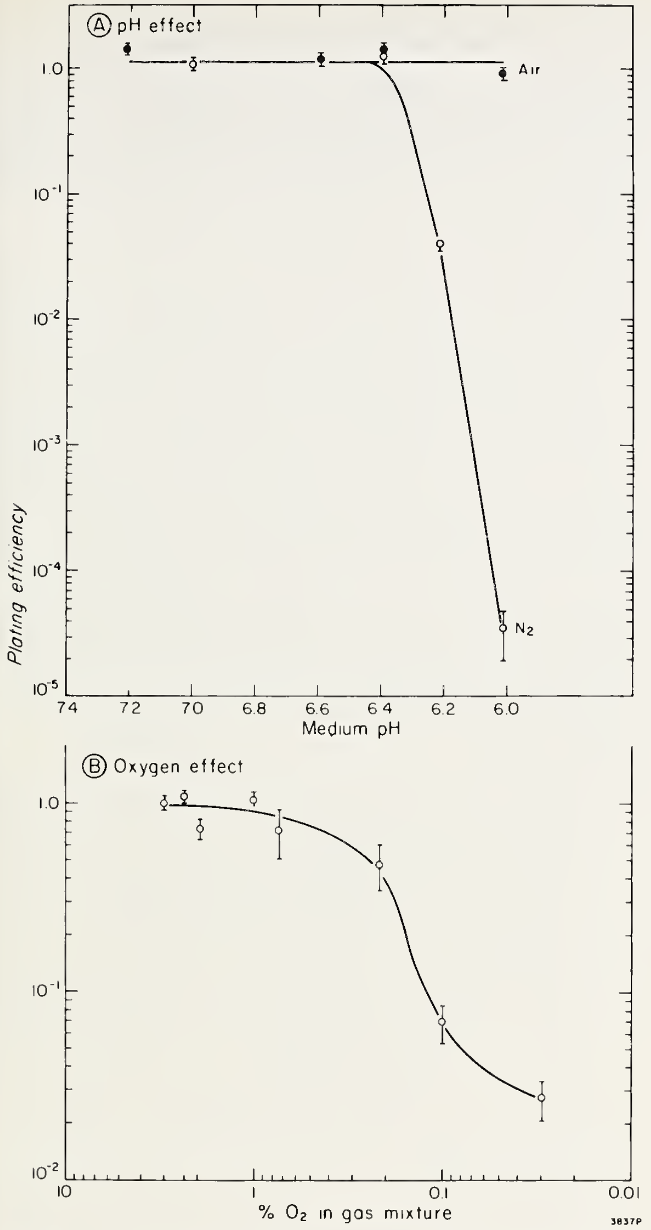


FIGURE 2.—A) Influence of pH on plating efficiency of CHO cells incubated under aerobic or hypoxic conditions for 6 hr. B) Influence of oxygen concentration on plating efficiency of CHO cells incubated for 6 hr at pH 6.2. Reprinted with permission from (*27*).

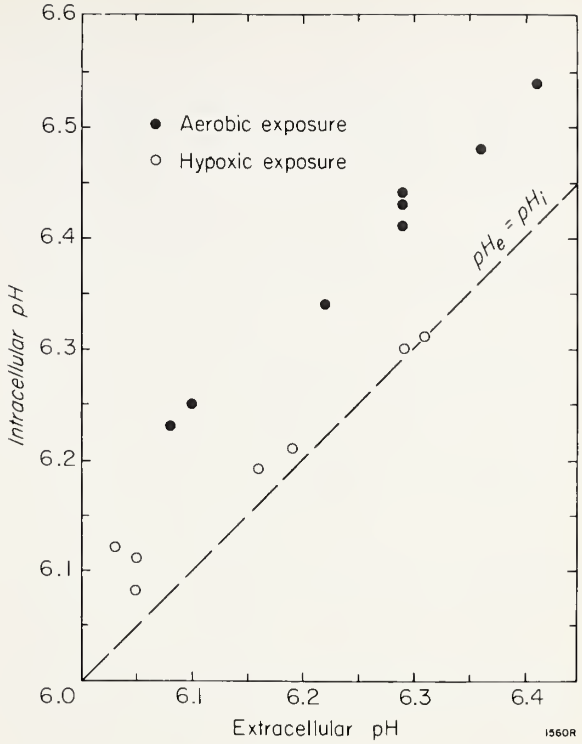


FIGURE 3.—Relationship between pH_e and pH_i for CHO cells incubated for 5 hr under aerobic or hypoxic conditions. pH_i was determined from distribution of the weak acid ^{14}C-5,5-dimethyl-2,4-oxazolidinedione (*28*).

(*21*). The mechanisms by which these drugs kill cells are currently under study, but measurement of pH_i by fluorimetry in the presence of the fluorescent probe 2,7-biscarboxyethyl-5(6)-carboxyfluorescein has confirmed that amiloride (fig. 6) and/or DIDS (data not shown) exacerbate the fall in pH_i caused by nigericin.

These results suggest the potential for using low tumor pH to achieve selective killing of cells. Nigericin and DIDS are both large molecules of limited solubility, which bind to protein, although they were active in the presence of serum, and may not be ideal candidates for cell killing in solid tumors. Nonetheless, if the mechanisms by which these effects can be achieved are elucidated, it may be possible to develop drugs with related structures that have optimal activity in vivo. If so, such drugs might be expected to give

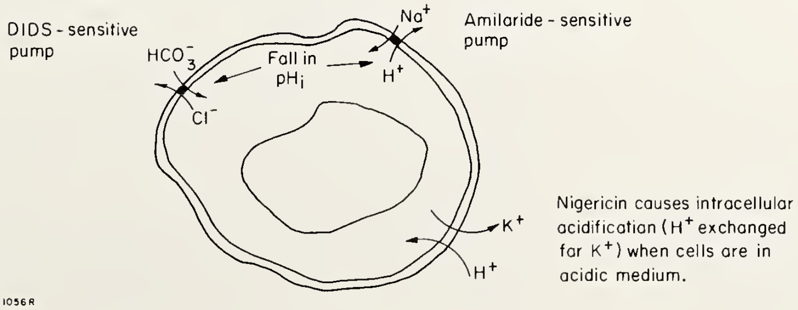

DIDS - sensitive pump
HCO₃⁻
Cl⁻
Fall in pHi
Na⁺
H⁺
Amilaride - sensitive pump
K⁺
H⁺
Nigericin causes intracellular acidification (H⁺ exchanged for K⁺) when cells are in acidic medium.
1056R

FIGURE 4.—Ion-exchange mechanisms in cell membrane that may act to regulate pH_i and their potential inhibition by drugs.

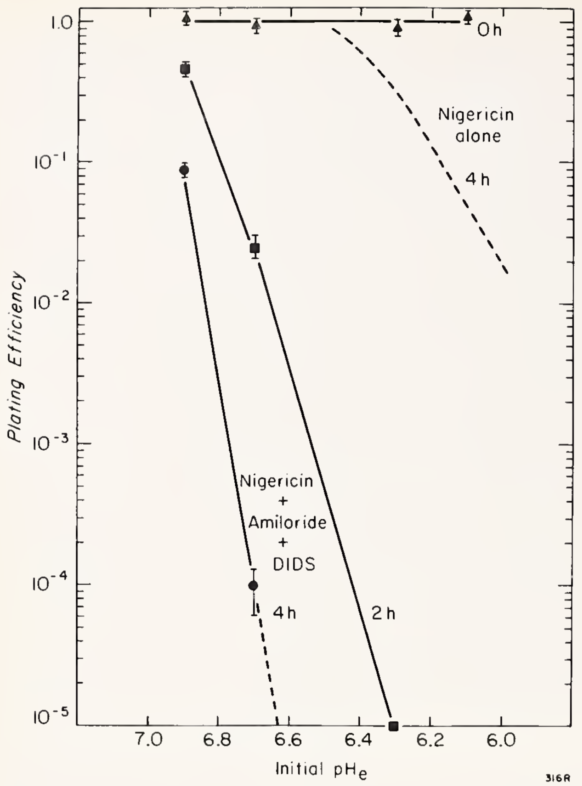


FIGURE 5.—Plating efficiency of CHO cells exposed at varying pH$_e$ to nigericin alone (0.25 μg/ml for 4 hr) or to combination of nigericin, amiloride (0.1 m*M*), and DIDS (0.1 m*M*) for 2 or 4 hr. Reprinted with permission from (*28*).

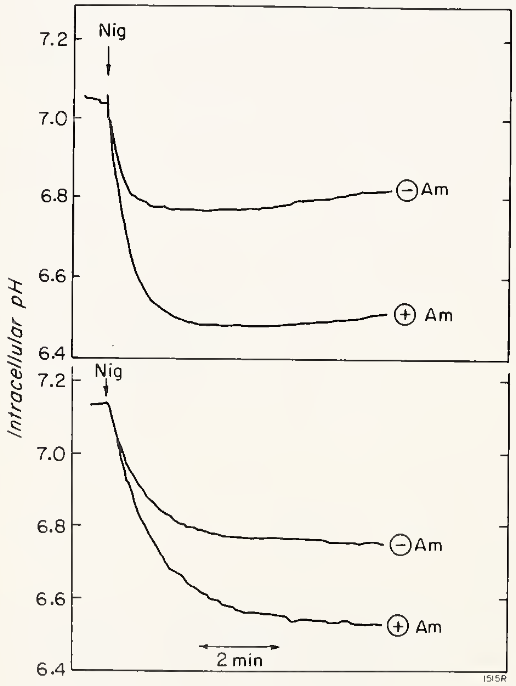


FIGURE 6.—Intracellular pH measured fluorimetrically using probe 2,7-biscarboxyethyl-5(6)-carboxyfluorescein for CHO cells (*upper*), and MGH-U1 cells (*lower*), following addition of nigericin (Nig; 0.25 μg/ml) with or without amiloride (Am; 0.1 m*M*). Extracellular pH was 6.77 at time drugs were added. Reprinted with permission from (*28*).

improved therapeutic effects when used with radiation. Our current research involves 1) testing these agents alone or with radiation in spheroids and experimental tumors; 2) studying the mechanism of action of these drugs, especially their effects on pH_i; and 3) studying related drugs for such properties as pH-dependent cell killing, protein binding and inactivation, and penetration into spheroids.

CONCLUSIONS

This brief review has emphasized that improvement in the therapeutic index through use of radiation and drugs is most likely to succeed if potential mechanisms can be proposed that would allow selective killing of tumor, compared to normal tissue, in the radiation field. Potential mechanisms are provided by the heterogeneity of cells that must be inactivated within tumors. This heterogeneity has multiple causes including genetic mutation, amplification or changes in expression of genes, factors related to cell differentiation, and environmental factors. Our studies of agents that might kill cells at acid pH but not at physiological pH are included as an example of how a known and variable property of cells in tumors might be used to design combined modality protocols that could lead to improvements in therapeutic outcome.

REFERENCES

(*1*) PHILLIPS TL (ed): Conference on Combined Modalities: Chemotherapy/Radiotherapy. Int J Radiat Oncol Biol Phys, vol 5 (No. 8–9), 1979.

(*2*) TANNOCK I: Cell kinetics and chemotherapy: A critical review. Cancer Treat Rep 62:1117–1133, 1978.

(*3*) HEPPNER GH: Tumor heterogeneity. Cancer Res 44:2259–2265, 1984.

(*4*) GOLDIE JH, COLDMAN AJ: The genetic origin of drug resistance in neoplasms: Implications for systemic therapy. Cancer Res 44:3643–3653, 1984.

(*5*) SCHIMKE RT: Gene amplification, drug resistance and cancer. Cancer Res 44:1735–1742, 1984.

(*6*) MCMILLAN TJ, STEPHENS TC, STEEL GG: Development of drug resistance in a murine mammary tumour. Br J Cancer 52:823–832, 1985.

(*7*) HILL RP, CHAMBERS AF, LING V, et al: Dynamic heterogeneity: Rapid generation of metastatic variants in mouse B16 melanoma cells. Science 224:998–1001, 1984.

(*8*) CILLO C, DICK JE, LING V, et al: Generation of drug-resistant variants in metastatic B16 mouse melanoma cell lines. Cancer Res. 47:2604–2608, 1987.

(*9*) WILSON RE, KENG PC, SUTHERLAND RM: Quantitation and kinetics of oxygen regulated protein induction. Radiat Res Soc Abstr 34:125, 1986.

(*10*) RICE GC, HOY C, SCHIMKE RT: Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. Proc Natl Acad Sci USA 83:5978–5982, 1986.

(*11*) HILL RP, STANLEY JA: The response of hypoxic B16 melanoma cells to in vivo treatment with chemotherapeutic agents. Cancer Res 35:1147–1153, 1975.

(*12*) DIXON B, MOORE JV, SPEAKMAN H: Radiobiological hypoxia of a transplanted rat tumour and the effect of treatment with cyclophosphamide. Eur J Cancer 14: 1383–1387, 1978.

(*13*) TANNOCK I: Response of aerobic and hypoxic cells in a solid tumor to Adriamycin and cyclophosphamide, and interaction of the drugs with radiation. Cancer Res 42:4921–4926, 1982.

(*14*) Chaplin DJ, Durand RE, Olive PL: Cell selection from a murine tumour using the fluorescent probe Hoechst 33342. Br J Cancer 51:569–572, 1985.

(*15*) Hill RP, Bush RS: A new method of determining the fraction of hypoxic cells in a transplantable murine sarcoma. Radiat Res 70:141–153, 1977.

(*16*) Tannock IF: Toxicity of 5-fluorouracil for aerobic and hypoxic cells in two murine tumors. Cancer Chemother Pharmacol 19:53–56, 1987.

(*17*) Durand RE: Chemosensitivity testing in V79 spheroids: Drug delivery and cellular microenvironment. JNCI 77:247–252, 1986.

(*18*) Siemann DW, Keng PC: Cell cycle specific toxicity of the Hoechst 33342 stain in untreated or irradiated murine tumor cells. Cancer Res 46:3556–3559, 1986.

(*19*) Hill RP, Gulyas S, Whitmore GF: Studies of the in vivo and in vitro cytotoxicity of the drug RSU 1069. Br J Cancer 53:743–751, 1986.

(*20*) Brown JM, Zeman EM, Baker MA, et al: SR 4233: A new bioreductive cytotoxic agent with high selective toxicity for hypoxic mammalian cells. Proc Am Assoc Cancer Res 27:285, 1986.

(*21*) Wike-Hooley JL, Haveman J, Reinhold JS: The relevance of tumor pH to the treatment of malignant disease. Radiother Oncol 2:343–366, 1984.

(*22*) Acker H, Holtermann G, Carlsson J, et al: pH gradients in glioma and thyroid cancer spheroids. Strahlentherapie 160:56, 1984.

(*23*) Tannock IF, Steel GG: Tumor growth and cell kinetics in chronically hypoxic animals. J Natl Cancer Inst 45: 123–133, 1970.

(*24*) Franko AJ, Sutherland RM: Rate of death of hypoxic cells in multicell spheroids. Radiat Res 76:561–572, 1978.

(*25*) Freyer JP, Sutherland RM: Regulation of growth saturation and development of necrosis in EMT6/Ro multicellular spheroids by the glucose and oxygen supply. Cancer Res 46:3504–3512, 1986.

(*26*) Tannock IF, Kopelyan I: Influence of glucose concentration on growth and formation of necrosis in spheroids derived from a human bladder cancer cell line. Cancer Res 46:3105–3110, 1986.

(*27*) Rotin D, Robinson B, Tannock IF: Influence of hypoxia and an acidic environment on the metabolism and viability of cultured cells: Potential implications for cell death in tumors. Cancer Res 46:2821–2826, 1986.

(*28*) Rotin D, Wan P, Grinstein S, et al: Cytotoxicity of compounds that interfere with the regulation of intracellular pH: A potential new class of anticancer drugs. Cancer Res 47:1497–1504, 1987.

(*29*) Tannock IF, Marshall N, Van Putten LM: An attempt at selective chemotherapy of hypoxic cells: Triethylenemelamine and irradiation of a C3H mouse mammary tumour. Eur J Cancer 8:501–507, 1972.

(*30*) Haveman J: The influence of pH on the survival after X-irradiation of cultured malignant cells. Effects of carbonylcyanide-3-chlorophenylhydrazone. Int J Radiat Biol 37:201–205, 1980.

Special Lecture: Alternating Chemotherapy and Radiotherapy[1]

William B. Looney[2,*]

ABSTRACT—The more effective utilization of well-established chemotherapeutic agents with radiotherapy can be brought to clinical use more rapidly and with greater efficiency than can new agents. New therapeutic approaches to improving cancer treatment will most likely be coordinated with either chemotherapy or radiotherapy or combined chemotherapy and radiotherapy. The results of experimental, theoretical, and clinical studies suggest that the potential for the greatest progress over the shortest period of time in the clinical management of tumors responsive to both treatment modalities lies in the more effective interaction of well-established chemotherapeutic agents with newer radiotherapy schedules in which large amounts of radiation can be delivered between the courses of chemotherapy and temporally separated to minimize toxicity without loss of therapeutic effectiveness. The experimental and theoretical studies reinforce the basic tenets first demonstrated in the clinical management of Hodgkin's disease. This provides the medical framework for more rapidly exploiting the concepts of alternating chemotherapy and radiotherapy in tumors responsive to both treatment modalities and brings options into sharper focus. 1) Either the doses given in radiotherapy or chemotherapy can be increased or the time between treatment modalities or between courses can be decreased. 2) Experimental, theoretical, and clinical results to date provide a rational basis for clinical protocols designed to determine the most effective utilization of these two primary means of cancer management.—NCI Monogr 6:85–94, 1988.

Clinical schedules for delivery of radiotherapy and chemotherapy have evolved independently. The underlying therapeutic strategy for the clinical utilization of chemotherapy has evolved from the necessity of using large doses separated by long time periods of 3–4 weeks, to allow primarily for gastrointestinal tract and bone marrow recovery. The underlying therapeutic strategy for the clinical utilization of radiotherapy has evolved from the necessity of using small daily radiation doses to avoid major damage to the normal tissue surrounding the tumor. However, there is no reason to believe that these methods of delivery represent optimal therapies. There are >100 different cancers. Clinical success to date is primarily the result of specific clinical protocols utilizing one or more treatment modalities to obtain an effective therapeutic ratio between the eradication of the specific tumor and the toxic effects on the host.

The major objective of the experimental cancer treatment program of our division was to provide a more rational and effective basis for the use of chemotherapy and radiotherapy. One of the most clinically relevant concepts that has evolved from our experimental studies is the superiority of alternating CP and radiotherapy in three courses of induction therapy (*1–3*). A cure rate $\geq 50\%$ was obtained in experimental solid tumors with this protocol, with acceptable toxicity to the host, as opposed to no cures from three or more courses of either modality alone at total doses that exceeded the permissible levels of damage to the host. Thus, disease that was unresponsive to one modality has been shown to be responsive to alternation of two modalities. The alternate use of chemotherapy and radiotherapy allows twice the recovery time from each modality and results in reductions in toxicity to within clinically acceptable limits for both modalities without decreasing tumor control and cure rates.

Hoppe et al. (*4*) at Stanford University (CA) in 1979 obtained a complete response rate of 87% from alternating chemotherapy and radiotherapy in patients with extensive Hodgkin's disease. They stated that this was the superior means of management and provides a model for combined modality therapy in patients with other malignancies. In 1984, Halberg et al. (*5*) presented a follow-up of the Stanford study and concluded that alternating chemotherapy and radiotherapy was superior to either chemotherapy alone, radiotherapy alone, or split-course radiotherapy and chemotherapy.

Our experimental studies are consistent with the basic tenet that has evolved from these clinical studies. The emphasis of our experimental studies has been aggressive induction therapy with the objective of cure. One of the major deterrents to tumor cure with combined chemotherapy and radiotherapy has been excessive toxicity. We have shown in our experimental system that temporal separation of the delivery of the two modalities is possible without loss of therapeutic effectiveness. The majority of clinical data show therapeutic additivity with combined chemotherapy and radiotherapy. Therefore, the results of studies to date indicate that toxicity can be reduced or minimized by temporal separation of delivery, thus circumventing one of the past major deterrents to the more effective clinical utilization of combined chemotherapy and radiotherapy.

ABBREVIATIONS: CP = cyclophosphamide; 5-FU = 5-fluorouracil; LD_{10} = dose(s) lethal to 10% of animals; T_{min} = time(s) at which minimum tumor volume occurred; T_{max} = time(s) at which maximum tumor growth rate occurred; DEF = dose-effect factor(s); MFD = multiple fractions per day; MOPP = mechlorethamine, vincristine, procarbazine, and prednisone.

[1] Supported in part by Public Health Service grants CA-20516 and CA-35372 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Division of Radiobiology and Biophysics, University of Virginia School of Medicine, Charlottesville.

* *Reprint requests:* William B. Looney, M.D., Division of Radiobiology and Biophysics, Box 392, University of Virginia Hospital, Charlottesville, VA 22908.

We believe that the potential for the greatest progress over the shortest period of time in the clinical management of tumors responsive to both treatment modalities lies in the more effective interaction of well-established chemotherapeutic agents with newer radiation schedules in which large amounts of radiation can be delivered between the courses of chemotherapy and temporally separated to minimize toxicity without loss of therapeutic effectiveness.

EXPERIMENTAL RESULTS

Our division was part of a multi-institution preclinical program on combined chemotherapy and radiotherapy initiated in 1976 by the Division of Cancer Treatment of the National Cancer Institute. In the beginning phases of our experimental treatment program, we evaluated the characteristics of six different tumors with different morphological, cell kinetic, biochemical, biologic, and growth (doubling times, 1–24 days) characteristics. From these six tumors, we selected two for the radiotherapy and chemotherapy program. One tumor, H-4-II-E, is resistant to radiation and to the representatives of three major classes of chemotherapeutic agents we have evaluated; i.e., 5-FU, doxorubicin, and CP. The other tumor, 3924A, is responsive to radiation and to these three chemotherapeutic agents. The experimental work presented in this review relates primarily to studies of the effects of radiation alone, CP alone, and the interaction of CP and radiation in hepatoma 3924A.

The effects of increasing single doses of CP alone were determined first (*6*). No cures were obtained when the dose was increased from 50 to 250 mg/kg (0.3–1.5 g/m^2). In rats, the intestinal mucosa recovers in approximately 6 days, but 10–12 days are required for recovery of bone marrow for single (LD_{10}) doses of 5-FU, doxorubicin, and CP. This bone marrow recovery time is approximately one-half of that in man (17–21 days) for comparable doses of cancer chemotherapeutic agents. There were no cures with CP in sequential (LD_{10}) doses of 150 mg/kg every 11 days for two, three, four, or five courses (total doses, 300–750 mg/kg) of CP. Three courses of CP (150 mg/kg) on days 0, 11, and 22 are optimal with regard to median survival in tumor-bearing rats.

The sequential administration of CP (150 mg/kg) every 11 days for four doses controlled tumor growth over the first 33 days of the study (fig. 1). Treatment resulted in a 46% increase in life span with no tumor cures. There was also an upward trend of mean tumor volume during the first 3 weeks following the sequential administration of a single dose of 1,500 rad of local tumor radiation every 11 days, as with CP.

Changes in mean tumor volume following administration of four series of alternating therapy (radiation, 1,500 rad; CP, 150 mg/kg) at 11-day intervals are shown in figure 2. There was complete response in 6 of the 9 animals given radiotherapy and chemotherapy alternately; at some point during the course of the experiment, no mass could be detected. There was a cure rate of 56%; 1 of the 6 tumors regrew. This experiment demonstrates that if large doses of either modality are given, long time periods between treatments are possible without a decrease in cure rates. The time interval between CP doses and between radiation doses was increased from 11 to 22 days. Thus, disease that did not respond to treatment with one modality is treatable by alternation of delivery of two modalities.

Daily irradiation with 250 rad/day for 6 days/week to a total dose of 9,000 rad prevented significant growth of hepatoma 3924A during the treatment period, but only one cure was achieved (fig. 3). The nadir for tumor reduction occurred 42 days after termination of treatment on day 40.

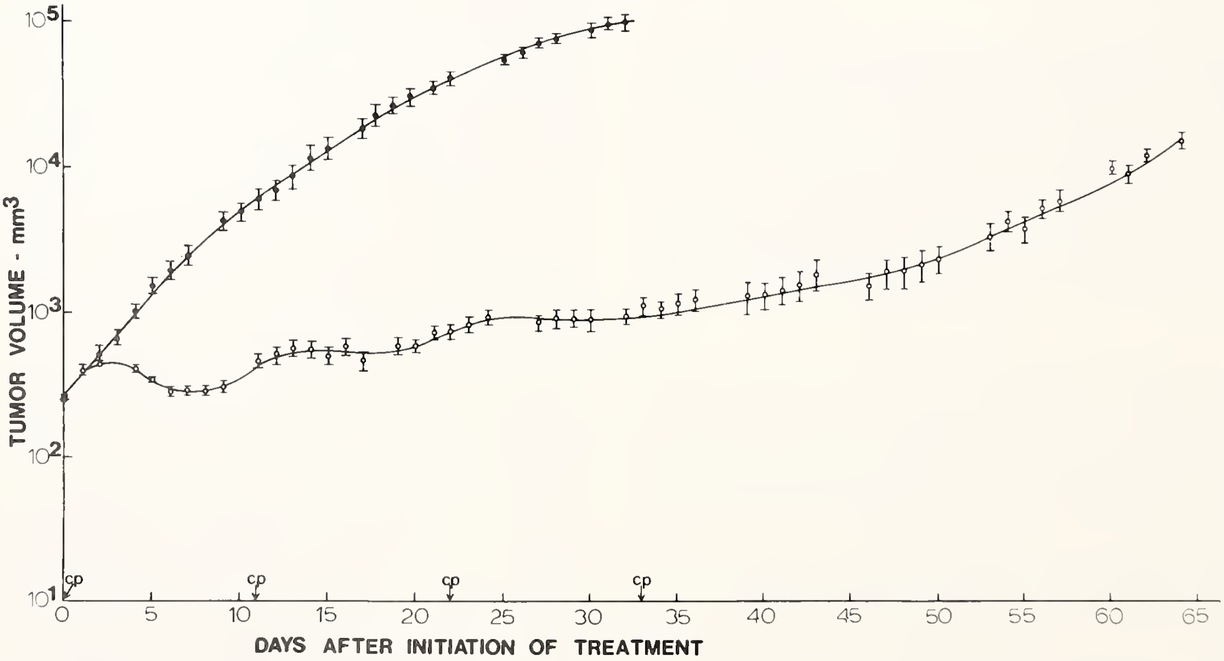


FIGURE 1.—Mean volume of 3924A tumors after sequential administration of CP (150 mg/kg) every 11 days for 4 doses. ○= treated; ●= control; *bars*=SE. Reprinted with permission from (*7*).

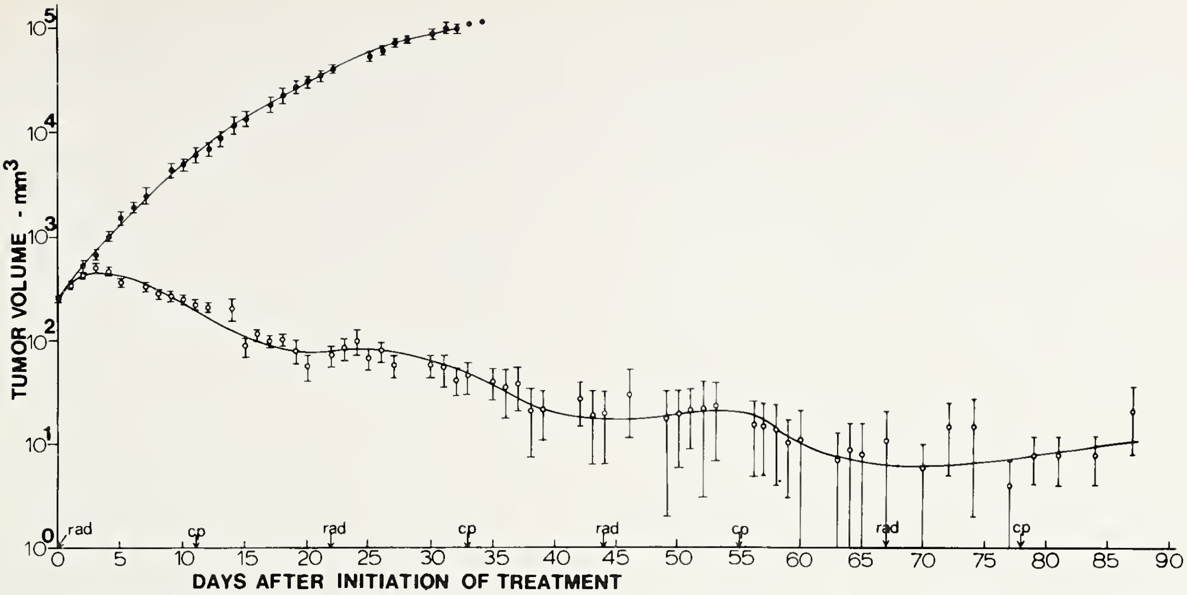


FIGURE 2.—Mean tumor volume for 4 series of combined modality therapy. Radiation (1,500 rad) and CP (150 mg/kg) were given at intervals of 11 days. ○=treated; ●=control; *bars*=SE. Reprinted with permission from (*7*).

This experimental schedule is similar to schedules of treatment 5 days/week for 6 weeks, which are used clinically.

The incorporation of [^{3}H]thymidine into tumor DNA has been used as an index for cellular proliferation. Incorporation of [^{3}H]thymidine into tumor DNA (3924A) during the 1-hour period prior to killing the animal is shown in figure 4. The DNA-specific activity in the CP-treated (150 mg/kg) tumors was depressed below control value for 4–5 days before recovery began; then it increased to a maximum at day 9.

Changes in tumor growth rate following four 150 mg/kg doses of CP given every 11 days are shown in figure 5. The T_{min} was 7 days after administration of each of the first three doses of CP and 5 days after the last dose of CP on day 33. The T_{max} occurred 10, 11, and 15 days after administration of CP on days 0, 11, and 22, respectively, and 26 days after administration of the fourth and final dose on day 33. The gradual reduction in growth rate of control tumors is a reflection of the slowing of tumor growth with increasing tumor size. Thus, biochemical and biomathe-

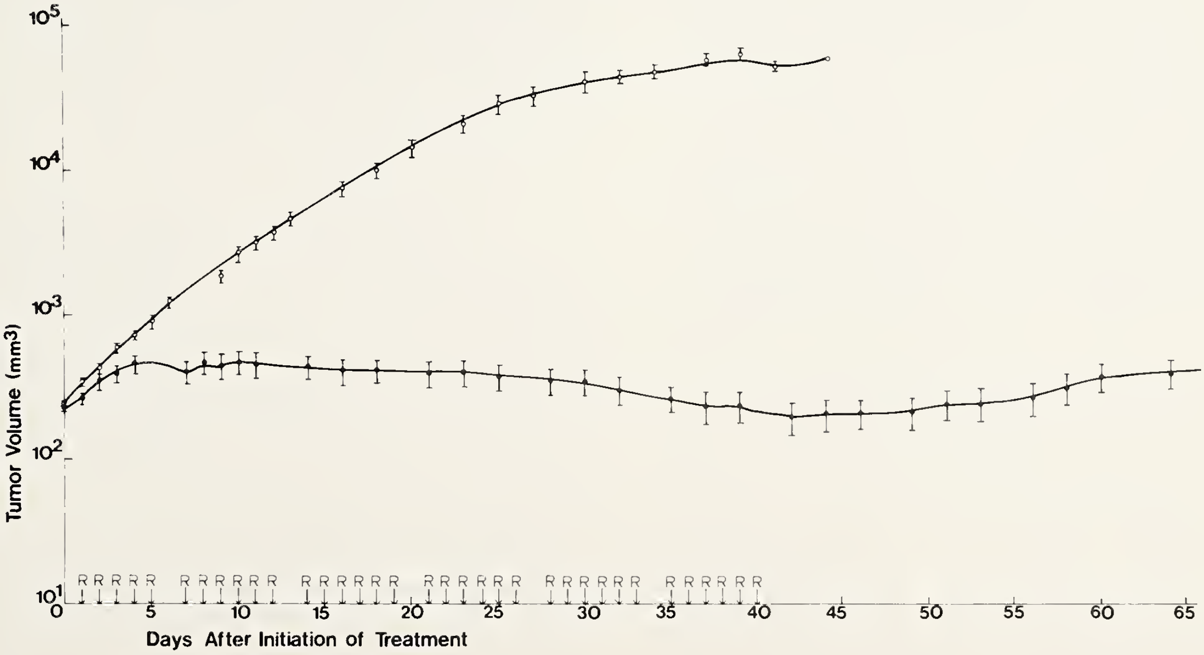


FIGURE 3.—Mean tumor volume after daily irradiation (R) (250 rad/day for 6 days/wk to total dose of 9,000 rad). ●=treated; ○ =control; *bars*=SE. Reprinted with permission from (*8*).

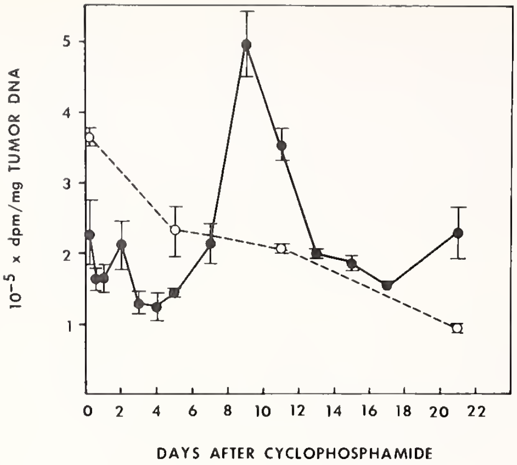


FIGURE 4.— Effect of CP (150 mg/kg) on [^{3}H]thymidine incorporation into DNA of hepatoma 3924A. ● = CP; ○ = control; *bars* = SE; dpm = disintegrations/min. Reprinted with permission from (*9*).

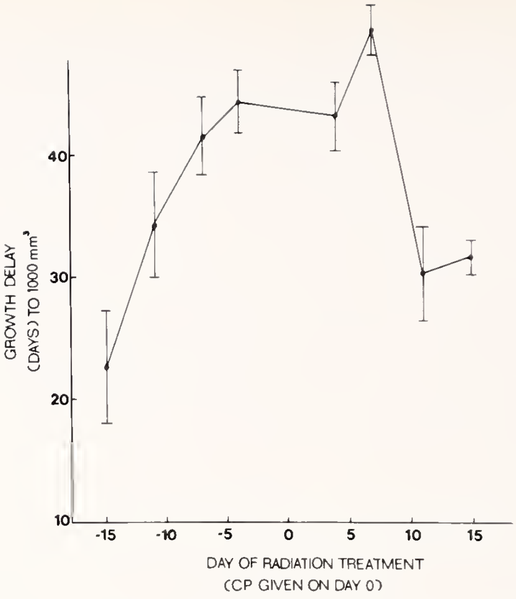


FIGURE 6.— Growth delay of 3924A tumors following combined treatment with radiation (1,500 rad) and CP (150 mg/kg). Treatments were separated by varing intervals of time as indicated. *Bars* = SE. Reprinted with permission from (*10*).

matical changes in growth rates following a major therapeutic insult from either chemotherapy or radiotherapy were confirmed by two independent methods. This phenomenon is not unique; it is a generalized response to therapeutic insult in different species including man (*11*).

To determine the time sequence for effective interaction of CP and radiation, the same radiation dose (1,500 rad) was given 15, 11, 7, or 4 days before CP (150 mg/kg) or 4, 7, 11, or 15 days after CP (fig. 6). The relative effectiveness of giving radiation before or after CP has been evaluated by tumor growth delay. Radiation given within 7 days of the CP (either before or after) produced the largest growth delays. Effectiveness was reduced when the interval between CP and radiation was increased to 11 or 15 days. Growth delays of 7.8 ± 1.87 (SE) days for radiation alone and 12.8 ± 0.49 days for CP alone have been achieved in previous studies. The ability to effectively interact the two

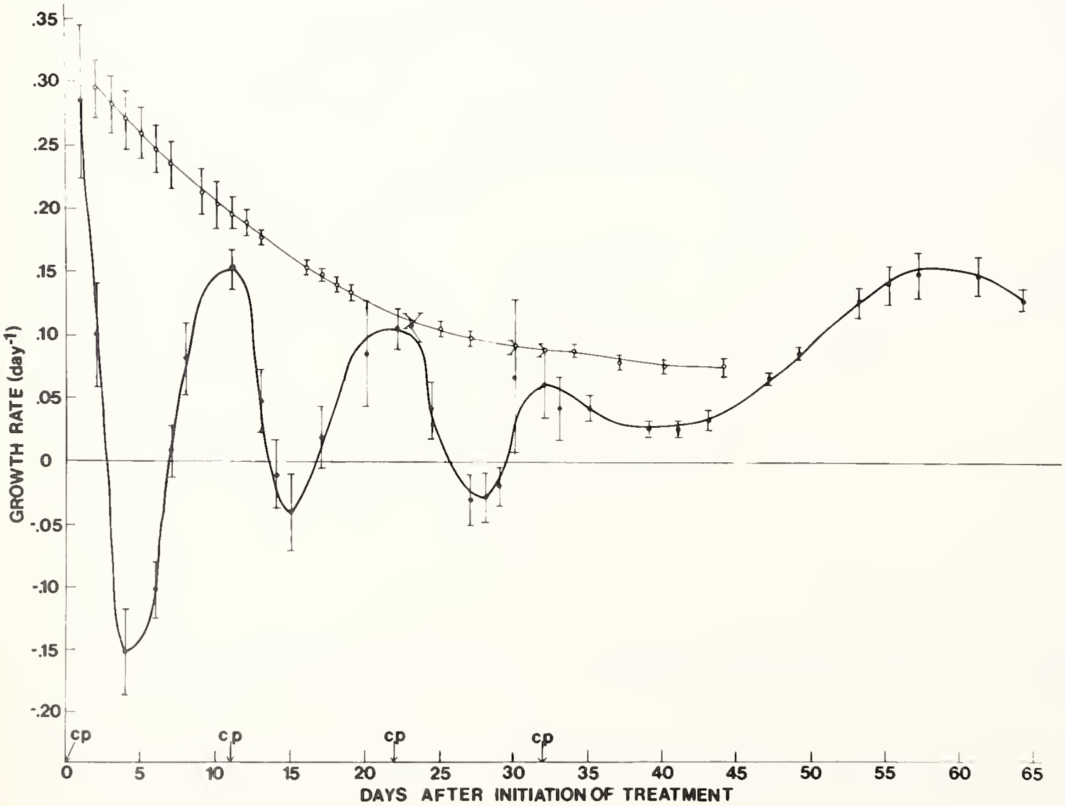


FIGURE 5.—Changes in tumor growth rate following 4 doses of CP (150 mg/kg). *Points* = mean of instantaneous growth rates for ten 3924A tumors on indicated day after treatment; *bars* = SE. ● = CP (150 mg/kg); ○ = control. Reprinted with permission from (*10*).

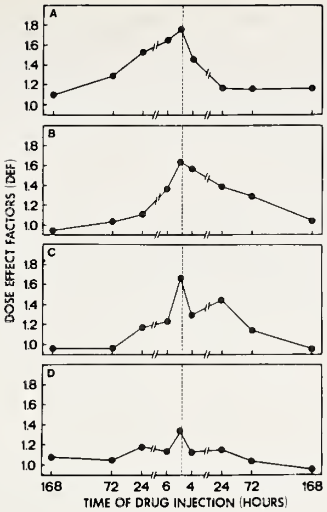


FIGURE 7.—DEF for A) doxorubicin; B) bleomycin; C) cyclophosphamide; and D) mitomycin administered from 7 days before (*left*) to 7 days after (*right*) irradiation. Reprinted with permission from (*12*).

modalities over a 7-day period has two therapeutic implications. It provides a 7-day interval for host recovery prior to giving the second modality, and it indicates that effective interaction of the two modalities does not depend on which agent is given first.

The investigators von der Maase et al. (*12*) determined changes in the DEF in mouse lung when different chemotherapeutic agents were given from 28 days before to 28 days after irradiation (fig. 7). Note the rapid reduction in DEF during 1–2 days before or after irradiation. This indicates that separation by 1 day can reduce lung toxicity when both modalities are given. These results are consistent with the results of our studies showing that large doses can be given within normal limits of tissue toxicity in both treatment modalities. The longer time period between delivery of alternate doses of each modality without loss of therapeutic effectiveness underscores one of the major advantages of this approach. It demonstrates that toxicity, which is one of the major clinical deterrents to giving combined modality treatment, may be avoided while the tumor is successfully treated.

The regimen for alternation of MFD irradiation with CP given 7 days after each of the three courses of MFD irradiation is shown in table 1. The cure rate was 70% following delivery of three courses over 35 days (total radiation dose, 7,500 rad) (*13*). No tumor cures were obtained after three courses of CP were given in combination with daily irradiation over 33 days (total radiation dose, 7,500 rad) (*2*). The rationale for MFD irradiation is that normal tissue recovers more rapidly than the tumor. Large doses of either radiation or CP can be given within 7 days before or after the other modality with little change in growth delay within this therapeutic window. The fraction size of 250 rad was used in subsequent experiments since the dose-response surface analysis of the data indicated that 250-rad fractions were optimal. This fraction size resulted in a >50% tumor cure rate with a minimum relative hazard when three courses of radiation were alternated with three courses of CP.

Goldie and co-workers (*14–16*) proposed that drug resistance results from somatic mutations in tumor cell populations and that "front-loaded," first-line treatment strategy with as many effective agents as possible in the highest dosages possible represents the clinician's best approach.

It is possible to provide a good fit between the predictions of the Goldie-Coldman model and the experimental results. Under the conditions described in this study (*17*), alternation of courses of chemotherapy and irradiation emerges as a powerful strategy capable of producing cures. The basis for this enhanced therapeutic effect is not completely understood, but the mathematical analysis suggests that an important component is the ability of each modality to extinguish subpopulations of tumor cells that are resistant to the other mode of therapy (*17*).

The superiority of alternating chemotherapy and radiotherapy over conventional regimens may be related to many factors other than reducing resistance. Some of these factors are: 1) an accelerated growth of the tumor starting about 7 days after the preceding therapeutic insult, which may increase the effectiveness of the subsequent treatment since increased therapeutic sensitivity has been observed in rapidly proliferating tumors; 2) the possibility of chemotherapy inhibiting the repair of sublethal or potentially lethal damage, occurring in hours; and 3) the inhibition of repair of chromosome damage, occurring in days.

Figure 8 shows tumor volumes for MFD irradiation given intermittently and alternated with CP (150 mg/kg) for three courses over a 35-day period, which achieved a cure rate of 70% (total radiation dose, 7,500 rad). Daily irradiation schedules are less effective than MFD schedules. The total radiation dose had to be increased to 11,250 rad when radiation was alternated with CP to achieve a cure rate of 50%. These data demonstrate the following:

1) same total doses of radiation (7,500 rad) and CP (450 mg/kg) for both experiments;
2) longer time of delivery with MFD versus daily irradiation (35 vs. 32 days);
3) cure rate of 70% for MFD irradiation given intermittently, as opposed to no cures for conventional daily irradiation schedules;
4) separation of 7 days for delivery of two modalities possible with increased therapeutic effectiveness for MFD over daily radiation alternated with CP;
5) importance of initial delivery of large doses of both treatment modalities in induction therapy if successful treatment is to be achieved;

TABLE 1.—Regimen for alternating chemotherapy and MFD radiotherapy[a]

Cycle	Day of cycle													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1st (days 1–14)	R	R						CP						
2nd (days 15–28)	R	R						CP						
3rd (days 29–42)	R	R						CP						

[a] Radiation (R) was given as 250 rad/fraction on days 1 and 2, 15 and 16, and 29 and 30 (total dose, 7,500 rad). CP (150 mg/kg or 0.9 g/m^2) was given on days 8, 22, and 36. CP was given 7 days after each of 3 courses of radiation; tumor cure rate = 70% (*13*).

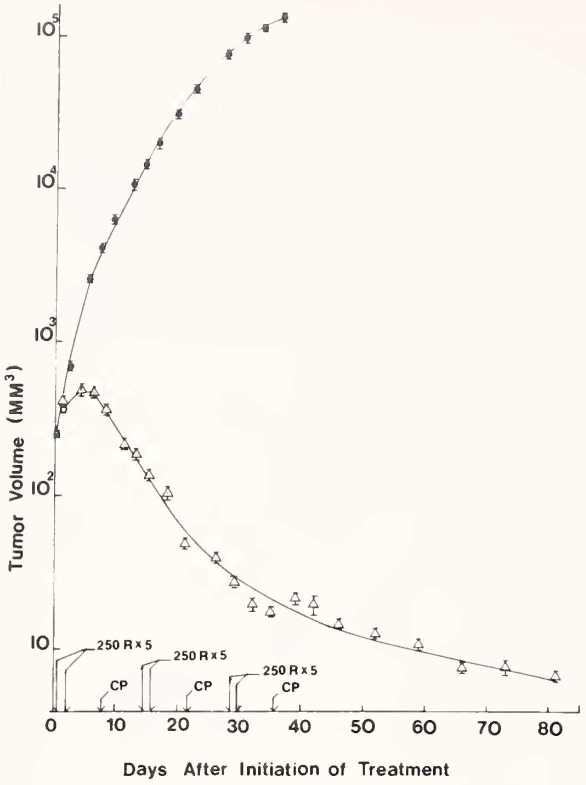


FIGURE 8.—Change in mean tumor volume following MFD irradiation in which CP was given 7 days after each of 3 courses of radiation (R) on days 0 and 1, 14 and 15, and 28 and 29. Total radiation dose was 7,500 rad, and 150 mg of CP/kg was administered on days 7, 21, and 35; ● = control; △ = radiation + CP.

6) importance of utilizing large doses in both modalities with the greatest intensity possible during the first two courses to avoid inadequate treatment and loss of tumor cures;
7) delivery of larger doses of radiation over shorter time intervals, allowing for longer recovery times between treatments; and
8) doubling of recovery time for each modality with alternation of chemotherapy and radiotherapy, thereby further enhancing the possibility of giving larger doses in each modality.

Tumor growth delay has been used to compare the relative effectiveness of different radiation dose schedules alone or in combination with CP. The data summarized in figure 9 demonstrate the following:

1) When radiation alone was administered either in daily fractions or as intermittent MFD, growth delays were comparable in relation to total radiation dose.
2) When CP was combined with radiation given in daily fractions, growth delay was additive for the two modalities.
3) When MFD schedules were combined with CP, growth delay was greater than additive. The relationship of growth delay to estimates of cell survival has been discussed elsewhere by Hopkins and Looney (*18*).

No complete responses or tumor cures occurred with radiation given daily for total radiation doses ≤11,250 rad or MFD given in intermittent doses up to a total dose of 9,000 rad. A cure rate of 50% was achieved with 6,000 rad when MFD irradiation was given intermittently and alternated with CP. A total dose of 11,250 rad was needed for a 50% cure rate when daily irradiation was alternated with CP.

CLINICAL RESULTS

The use of well-established chemotherapeutic agents in the treatment of tumors known to be responsive has a number of advantages over attempting new and clinically untested agents with radiation. Most effective chemotherapeutic agents or combinations of agents have established response rates so that these therapeutic results can be utilized in the design of clinical protocols.

This is illustrated in the clinical protocol of alternating MOPP with radiation in the clinical management of patients with extensive Hodgkin's disease (*4*). Giving both modalities together resulted in excessive toxicity. Among the complete responders, the 5-year survival rate is 98%, and 96% are free from relapse. The median follow-up of survivors is 4.6 years (range, 1.3–13). Alternating chemotherapy and radiotherapy is now the standard method of treatment of extensive Hodgkin's disease at Stanford (table 2).

The improvement in outcome for patients with advanced disease treated in the various Stanford clinical trials (*19*) is shown in figure 10; freedom from relapse and survival is given for patients with stage IIIB disease. Patients treated with total lymph node irradiation alone had a very poor outcome: only 15% are long-term disease-free survivors (fig. 10, No. 3). In contrast, >50% of the patients treated with total lymph node irradiation followed by MOPP are long-term disease-free survivors (fig. 10, No. 2). This result, however, is little better than could be expected after treatment with MOPP alone. In contrast, the alternating program of chemotherapy and radiotherapy has proved superior to these other approaches, with 76% of the patients enjoying long-term disease-free survival (fig. 10, No. 1).

Arcangeli and associates (*20*) have demonstrated that it is

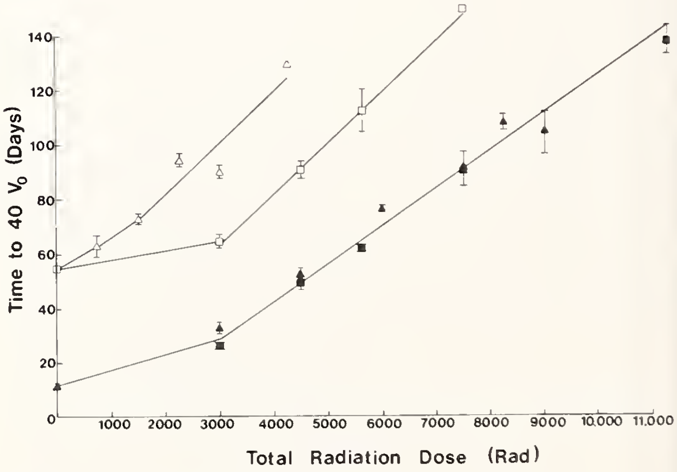


FIGURE 9.—Mean time required for tumor volume to reach 40 times initial volume at time of treatment (40 V_0) for increasing total doses of radiation given in daily fractions (■) or as MFD alone (▲); in daily fractions + CP (⊡); or in MFD + CP (△).

TABLE 2.—Schema of alternating chemotherapy and RT used at Stanford in treatment of Hodgkin's disease[a]

	Day	
Month	1 2 3 4 5 6 7 8 9 10 11 12 13 14	15 16 17 18 19 20 21 22 23 24 25 26 27 28
1	← MOPP →	
2	← MOPP →	
3		← RT to most involved areas →[b]
4	← RT continued to most involved areas →	
5	← MOPP →	
6	← MOPP →	
7		← RT to 2nd involved area →[b]
8	← RT continued to 2nd involved area →	
9	← MOPP →	
10	← MOPP →	
11		← RT to least involved area →[b,c]
12	← RT continued to least involved area →	

[a] Chemotherapy with MOPP consisted of mechlorethamine (6 mg/m^2) on days 1 and 8; vincristine (1 mg/m^2) on days 1 and 8; procarbazine (100 mg/m^2) on days 1–14; and prednisone (40 mg/m^2) on days 1–14.

[b] Radiotherapy may start as early as day 1 of month 3 for this 1st treatment field; patients received 150–200 rad/day for 5 days/wk to 4,400 rad (*4*).

[c] Dose was 3,000 rad to uninvolved areas.

possible to give intermittent MFD irradiation alternated with chemotherapy to patients with stage III non-small cell lung cancer with promising therapeutic response and minimal or no toxic effects. On these experimental bases, a protocol was designed using MFD split-course radiotherapy alternated with chemotherapy; it was tested in a pilot study of 52 patients with stage III non-small cell lung cancer. Radiotherapy was given as three fractions of 200 rad on days 1 and 2, 8 and 9, 22 and 23, and 29 and 30, for a total dose of 4,800 rad. A three-drug combination of CP (400 mg/m^2), doxorubicin (17 mg/m^2), and methotrexate (15 mg/m^2) was given on days 3 and 24 and was repeated every 21 days.

An improvement of performance status was observed in 22 patients previously assigned to categories ≤80. Furthermore, 6 patients (11%) achieved complete response, and 29 (56%) had partial response. Minor response or disease progression was observed in the remaining 17 patients (33%). The median life span was 41 weeks. The estimated median life spans for responding and nonresponding patients were 61 and 28 weeks, respectively.

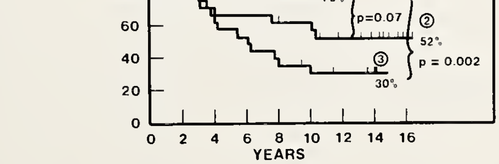


FIGURE 10.—Comparison of sequential Stanford clinical trials for management of stage IIIB Hodgkin's disease. Reprinted with permission from (*19*).

The majority of patients exhibited very mild or no side effects after treatment. The World Health Organization grades were predominantly 0 or I. These preliminary results confirm the possibility of achieving a therapeutic gain by alternating MFD radiotherapy and chemotherapy, especially in the instance of patients with localized disease in which radiation doses can be delivered to the entire tumor.

The Institut Gustave Roussy (Villejuif, France) reported in 1985 (*21,22*) that radiotherapy and chemotherapy can be separated by 7 days and alternated to produce a complete response rate of 89% and a relapse-free survival of 28% at 3 years, within acceptable limits of toxicity in patients with limited small cell lung carcinoma (table 3). Therefore, both clinical and experimental results indicate that excessive toxicity resulting from giving both modalities together can be avoided by temporal separation. This indicates that one of the major deterrents for effectively using combined chemotherapy can be circumvented by simply temporally separating the delivery of the two modalities and alternating the administration to provide twice the recovery time for normal tissue for each modality. There is no clinical evidence to date to suggest that effective doses of each modality cannot be given if they are temporally separated. Therefore, it is feasible to utilize proven and effective doses of both modalities.

A comparison is made in table 4 of the results of alternating chemotherapy and radiotherapy by giving radiotherapy after six courses of chemotherapy or giving chemotherapy and radiotherapy concomitantly for several clinical studies. The results of the Institut Gustave Roussy (*22*) using alternating chemotherapy and radiotherapy are better than the results of giving all six courses of chemotherapy prior to radiotherapy at the Ontario Cancer Institute (Toronto, Canada) (*23*). There was a complete response rate of 89% in the study of Arriagada et al. (*22*), compared to 52% in the study of Feld et al. (*23*) (table 3). The median survival was 84 weeks, compared with 49 weeks, respectively. Furthermore, the relapse-free survival at 2 years was 37% in the French study (*22*), compared to 19% in the Canadian study

TABLE 3.—Schema of alternating chemotherapy and RT used at Institut Gustave Roussy in treatment of small cell lung carcinoma[a]

	Day		
Month	1 2 3 4 5 6	7 8 9 10 11 12	13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
1	←Chemotherapy→		
2	←Chemotherapy→	RT1	←3,000 rad to brain[b] → 10 fractions/12 days
		RT1	←2,000 rad to tumor mass, mediastinum, and supraclavicular nodes[c] → 8 fractions/12 days
3	←Chemotherapy→	RT2	←2,000 rad to tumor mass, mediastinum, and supraclavicular nodes[c] → 8 fractions/12 days
4	←Chemotherapy→	RT3	←1,500 rad to tumor mass, mediastinum, and supraclavicular nodes[c] → 6 fractions/10 days
5	←Chemotherapy→		

[a] Chemotherapy consisted of doxorubicin (40 mg/m^2) on day 1; CP (300 mg/m^2) on days 3–6; etoposide (75 mg/m^2) on days 1–3; and cisplatin (100 mg/m^2) on day 2. Radiotherapy=RT1, RT2, and RT3 as 1st, 2nd, and 3rd courses, respectively.

[b] Therapy was given in 300-rad fractions.

[c] Therapy was given in four 250-rad fractions weekly.

(*23*). The relapse-free survival at 3 years was 28%, compared to a projected 10% in the study of Feld et al. (*23*). These clinical study results are comparable to our experimental results using alternating chemotherapy and radiotherapy versus the administration of all the chemotherapy prior to giving radiotherapy.

The results of Lichter et al. (*24*) at the National Cancer Institute (Bethesda, MD) are intermediate between those of the French and Canadian studies. The complete response rate was 78% (table 3), and the median disease-free interval was 68 weeks. The better results in the study by Lichter et al. compared to the study by Feld et al. (*23*) probably reflect the outcome of giving the radiation early with the chemotherapy as well as a larger total radiation dose (4,000 vs. 2,500 rad). The major disadvantage in the study by Lichter et al. is the increased toxicity that occurs when the two modalities are given concurrently. This toxicity will probably limit the total radiotherapy and chemotherapy doses that can be administered during induction therapy.

CONCLUSIONS

Experimental Studies

Experimental studies have shown the superiority of alternating chemotherapy and radiotherapy during induction therapy over sequential or concomitant utilization.

1) A cure rate ≥50% was obtained when CP was alternated with radiation, with acceptable toxicity to the host.
2) No cures were obtained with either modality alone or the sequential use of both modalities.
3) Thus, disease that was unresponsive to one modality has been shown to be responsive to alternation of the two modalities.
4) The major deterrent to clinical utilization of both modalities given together has been excessive toxicity. Experimental results have demonstrated that one modality can be given within 7 days before or after the other modality without loss of therapeutic effectiveness. This temporal separation reduces toxicity. In addition, alternation of modalities allows for twice the recovery time for each modality, further reducing toxicity.

Theoretical Studies

Goldie and co-workers (*14–16*) have proposed that drug resistance results from somatic mutations in tumor cell populations and that front-loaded, first-line treatment strategy with as many effective agents as possible in the highest dosages possible represents the clinician's best approach. The mathematical model developed by Goldie and Coldman (*15*) relates the drug sensitivity of a tumor to its own spontaneous mutation rate toward phenotypic drug resistance. The basic assumption is that tumors are curable by chemotherapy if no permanently resistant cell lines are present.

It is possible to provide a good fit between the predictions of the Goldie-Coldman model and the experimental results. Under the conditions described in this study, an approach using alternating courses of chemotherapy and radiotherapy emerges as a powerful strategy capable of

TABLE 4.—Results of chemotherapy and radiotherapy for limited small cell lung carcinoma

Treatment	Institution	Investigators (ref. No.)	No. of patients	Complete response (%)	Median survival (wk)	Relapse-free survival (%)	
						2 yr	3 yr
Chemotherapy + radiotherapy							
Alternated	Institut Gustave Roussy	Tubiana et al. (*21*)	45	89	84	37	28
Sequential[a]	Ontario Cancer Institute	Feld et al. (*23*)	153	52	49	19	10[b]
Concomitant	National Cancer Institute	Lichter et al. (*24*)	37	78			

[a] All chemotherapy given before radiotherapy.

[b] Projected.

producing cures. The basis for this enhanced therapeutic effect is not completely understood, but the mathematical analysis suggests that an important component is the ability of each modality to extinguish subpopulations of tumor cells that are resistant to the other mode of therapy.

Clinical Studies

In 1979, Hoppe et al. (*4*) at Stanford, reporting a complete response rate of 87%, stated that alternating chemotherapy and radiotherapy in patients with extensive Hodgkin's disease was the superior means of management and provides a model for combined modality therapy in patients with other malignancies. A follow-up by Halberg et al. (*5*) in 1984 concluded that alternating chemotherapy and radiotherapy was superior to either chemotherapy alone, radiotherapy alone, or split-course radiotherapy and chemotherapy.

Tubiana and associates (*21*) at the Institut Gustave Roussy have been using alternating chemotherapy and radiotherapy in the treatment of limited small cell carcinoma of the lung since 1980. They are finding a complete tumor response rate of 89% with a relapse-free survival rate of 25% at 4 years. The clinical implications of this study are that in 1 year, if these results are maintained, approximately 25% of these patients can be considered cured on the basis of 5-year survival criteria.

Relationship of Theoretical and Experimental Results to Clinical Studies

Dr. Vincent T. DeVita, Jr., director of the National Cancer Institute, reported at the 1986 American Association of Cancer Research meeting (*25*) that inadequate treatment of Hodgkin's disease and large cell lymphoma results in up to 4,000 deaths each year in the United States. We have shown experimentally that the first two courses of alternating chemotherapy and radiotherapy given with the greatest intensity possible within limits of normal tissue toxicity are crucial to therapeutic success (i.e., tumor cure rates ≥50%). These experimental and theoretical studies address the important clinical question raised by Dr. DeVita of giving chemotherapy and radiotherapy initially with maximum intensity within limits of normal tissue toxicity to avoid inadequate induction therapy.

Dr. DeVita (*26*) has summarized concepts relating to the development of resistance to chemotherapy. He concludes that it is obvious that more effective anticancer drugs are still required to maximally exploit our current knowledge in cancer chemotherapy but that it also seems likely that more can be achieved with current drugs.

The experimental and theoretical studies reinforce the basic tenets first demonstrated in the clinical management of Hodgkin's disease. This provides the medical framework for more rapid exploitation of the concept of alternating chemotherapy and radiotherapy in tumors responsive to both treatment modalities. Clinical protocols giving radiation and chemotherapy alternately, sequentially, or intermittently discussed in this conference do not differentiate clinically which is best.

Experimental and theoretical data for alternating chemotherapy and radiotherapy bring options into sharper focus. 1) Either the dosages given for radiotherapy or chemotherapy can be increased or the time between treatment modalities or between courses can be decreased. 2) The clinical, experimental, and theoretical results to date provide a rational basis for clinical protocols designed to determine the most effective utilization of these two primary means of cancer management.

The more effective utilization of well-established chemotherapeutic agents with radiotherapy can be brought to clinical use more rapidly and with greater efficiency than can new agents. New therapeutic approaches to improving cancer treatment will most likely be coordinated with either chemotherapy or radiotherapy or combined chemotherapy and radiotherapy. We believe that the potential for the greatest progress over the shortest period of time in the clinical management of tumors responsive to both treatment modalities lies in the more effective interaction of well-established chemotherapeutic agents with newer irradiation schedules in which large amounts of radiation can be delivered between the courses of chemotherapy and temporally separated to minimize toxicity without loss of therapeutic effectiveness.

REFERENCES

(*1*) Looney WB, Hopkins HA: Alternation of chemotherapy and radiotherapy in cancer management. III. Results in experimental solid tumor systems and their relationship to clinical studies. Cancer Treat Rep 70:141–162, 1986.

(*2*) Looney WB, Hopkins HA, Carter WH Jr: Solid tumor models for the assessment of different treatment modalities. XXIII. A new approach to the more effective utilization of radiotherapy alternated with chemotherapy. Int J Radiat Oncol Biol Phys 11:2105–2117, 1985.

(*3*) Looney WB, Hopkins HA, Kovacs CJ, et al: Single and combined (radiation-cyclophosphamide) modality therapy in experimental solid tumors. *In* Advances in Radiation Biology (Lett J, ed). New York: Academic Press, 1983, pp 306–402.

(*4*) Hoppe RT, Portlock CS, Glatstein E, et al: Alternating chemotherapy and irradiation in the treatment of advanced Hodgkin's disease. Cancer 43:472–481, 1979.

(*5*) Halberg FE, Hoppe RT, Rosenberg SA, et al: Alternating chemotherapy and irradiation in advanced Hodgkin's disease. Int J Radiat Oncol Biol Phys 10(suppl 2):105, 1984.

(*6*) Looney WB, Ritenour ER, Hopkins HA: Changes in growth rate of an experimental solid tumor following increasing doses of cyclophosphamide. Cancer Res 40:2179–2183, 1980.

(*7*) Looney WB, Ritenour ER, Hopkins HA: Solid tumor models for the assessment of different treatment modalities. XIX. Tumor cure rates and tumor control following sequential administration of radiation and cyclophosphamide. Am J Clin Oncol 5:209–220, 1982.

(*8*) Looney WB, Hopkins HA, Longerbeam MB, et al: Comparison of effects of daily versus hyperfractionated split-course radiation schedules with and without cyclophosphamide on median survival, metastatic dissemination, tumor cure, and growth rates. Cancer Res 43:60–67, 1983.

(*9*) Looney WB, Hopkins HA, Grover WH, et al: Solid tumor models for the assessment of different treatment modalities. XIII. Comparison of response and recovery of host and solid tumor to cyclophosphamide and radiation. Cancer 45:2793–2804, 1980.

(*10*) Looney WB, Hopkins HA: Solid tumors as a model for the development of antineoplastic therapy. *In* Methods in Cancer Research (Busch H, Yeoman LC, eds), vol XIX. New York: Academic Press, 1982, pp 303–384.

(*11*) Van Peperzeel HA: Effects of single doses of radiation on lung metastases in man and experimental animals. Eur J Cancer 8:665–675, 1972.

(*12*) von der Maase H, Overgaard J, Vaeth M: Effect of cancer chemotherapeutic drugs on radiation-induced lung damage in mice. Radiother Oncol 5:245–257, 1986.

(*13*) Looney WB, Hopkins HA, Carter WH Jr: Solid tumor models for the assessment of different treatment modalities. XXII. The alternate utilization of radiotherapy and chemotherapy. Cancer 54:416–425, 1984.

(*14*) Goldie JH: New thoughts on resistance to chemotherapy. Hosp Pract 18:165–177, 1983.

(*15*) Goldie JH, Coldman AJ: A mathematic model for relating the drug sensitivity of tumors to their spontaneous mutation rate. Cancer Treat Rep 63:1727–1733, 1979.

(*16*) Goldie JH, Coldman AJ, Gudauskas GA: Rationale for the use of alternating non-cross-resistant chemotherapy. Cancer Treat Rep 66:439–449, 1982.

(*17*) Goldie JH, Coldman AJ, Ng V, et al: A mathematical and computer based model of alternating chemotherapy and radiation therapy in experimental neoplasms. Lung Cancer. In press.

(*18*) Hopkins HA, Looney WB: Solid tumor models for the assessment of different treatment modalities. XXVI. Estimates of cell survival from tumor growth delay after alternating radiotherapy and chemotherapy. Int J Radiat Oncol Biol Phys 13:217–224, 1987.

(*19*) Hoppe RT, Horning SJ, Rosenberg SA: The concept, evolution and preliminary results of the current Stanford clinical trials for Hodgkin's disease. Cancer Surv 4:459–475, 1985.

(*20*) Arcangeli G, Righini R, Nervi C, et al: Pilot study of multiple-fraction daily radiotherapy alternating with chemotherapy in patients with stage IV non-oat cell lung cancer. Cancer Treat Rep 69:25–31, 1985.

(*21*) Tubiana M, Arriagada R, Cosset JM: Sequencing of drugs and radiation. The integrated alternating regimen. Cancer 55(suppl 9):2131–2139, 1985.

(*22*) Arriagada R, Le Chevalier T, Baldeyrou P, et al: Alternating radiotherapy and chemotherapy schedules in small cell lung cancer, limited disease. Int J Radiat Oncol Biol Phys 11:1461–1468, 1985.

(*23*) Feld R, Evans WK, DeBoer G, et al: Combined modality induction therapy without maintenance chemotherapy for small cell carcinoma of the lung. J Clin Oncol 2:294–304, 1984.

(*24*) Lichter AS, Bunn PA, Ihde DC, et al: The role of radiation therapy in the treatment of small cell lung cancer. Cancer 55:2163–2175, 1985.

(*25*) DeVita VT Jr: Chemotherapy of the lymphomas: Looking back—Moving forward. The 10th Richard and Hinda Rosenthal Foundation Award Lecture. Presented at the Annual Meeting of the American Association for Cancer Research, Los Angeles, May 7–10, 1986.

(*26*) DeVita VT: Some new concepts on the development of resistance to chemotherapy. *In* Accomplishments in Cancer Research 1982 (Fortner JG, Rhoads JE, eds). Philadelphia: Lippincott, 1983, pp 99–119.

Response of Cell Subpopulations in Spheroids to Radiation-Drug Combinations[1]

Ralph E. Durand* and Sandra L. Vanderbyl[2,3]

ABSTRACT—Chinese hamster V79 cells grown as multicell spheroids were treated with radiation and/or selected antineoplastic drugs (5-fluorouracil, cisplatin, doxorubicin, mitomycin, and carmustine) administered at equitoxic levels in defined preirradiation and postirradiation sequences. Clonogenicity of the treated cells was determined, and the results from conventional assays were compared with those from cell-selection experiments, using a fluorescence-activated cell sorter, to determine the degree of interaction and regions of activity of the agents. Marked sequence-dependent differences in overall toxicity were observed; drug toxicity, spheroid reoxygenation, and direct sensitization appeared to be operative to a different extent with each drug and sequence. Maximal interaction between all drugs and radiation was, however, observed when drug exposure immediately preceded irradiation.—NCI Monogr 6:95–100, 1988.

The development of effective chemotherapeutic agents for human cancers has led to a natural desire to integrate chemotherapy treatment with other modalities, especially radiotherapy. Clinical protocols of this type are generally based on two different philosophic approaches: "spatial cooperation" (i.e., radiotherapy for the primary tumor and chemotherapy for metastatic disease) or "radiosensitization" (i.e., increased efficacy of radiation for local control). Therapeutic ratio plays a critical role in radiosensitization because increases in antitumor effect must be greater than increases in toxicity to the surrounding normal tissues.

Unfortunately, as indicated in several recent reviews (*1,2*), the toxicity of combined chemotherapy-radiotherapy treatment is a major obstacle in the clinic. From the laboratory perspective, however, this toxicity seems to be irrefutable evidence that conventional antineoplastic agents *can* act as radiosensitizers; it suggests that effective clinical utilization of chemotherapy-radiotherapy combinations may be largely a question of more advantageous dosages and sequences. When drugs and radiation are combined, sequencing effects can result from cytotoxic interactions or radiosensitizing properties of the drug or through secondary mechanisms. The chemotherapeutic agent may, for example, cause a microenvironmental change like reoxygenation, which sensitizes the previously hypoxic tumor cells. Consequently, a first step toward more effective clinical usage of these modalities is a better understanding of the various methods by which they can interact.

Chinese hamster V79 spheroids are a useful model to investigate sequencing effects of radiation-drug combinations. They display many tumorlike properties and have morphology similar to that of the nodular areas of many solid tumors (*3,4*). The growth fraction of cells in spheroids decreases with time as it does in many tumors (*5*), concurrent with karyotypic instability (*6*) and the development of large numbers of noncycling cells. Nutrient and oxygen supplies are dependent on diffusion inward from the periphery, so tumorlike central necrosis and hypoxia spontaneously develop in larger spheroids (*7,8*). Cell viability can be measured using conventional assays of clonogenicity; additionally, we have developed techniques of fluorescence staining and fluorescence-activated cell sorting (*9–11*) to accurately measure the drug or radiation response of cells from *known* positions in the spheroid. Radiation-drug sequence effects can thus be determined easily in spheroids, independently of the metabolic, immunologic, or other tumor-host interactions of animal systems.

In this report, five drugs used in cancer chemotherapy (5-FU, cisplatin, doxorubicin, mitomycin, and carmustine), chosen as representative of different drug classes effective in a broad spectrum of tumor sites, were administered to spheroids in preirradiation or postirradiation sequences. Administration of the drug after radiation exposure and comparison of the observed response to that expected for independent action of the two agents provide an estimate of the role of differential drug toxicity. If both agents kill the same cell subpopulations, less total toxicity is expected than if different subpopulations are targeted. Conversely, direct modification of the radiosensitivity of the various spheroid subpopulations was studied by giving irradiation during the last few minutes of drug exposure. Comparison of these results with those of a protocol in which the drug-treated spheroids were returned to standard growth conditions for a 3-hour period prior to irradiation allowed separation of the transient metabolic effects present during drug exposure from the more persistent effects of cell death.

Virtually equitoxic exposures to the five drugs produced marked, sequence-dependent differences in overall toxicity when combined with radiation; drug toxicity, spheroid reoxygenation, and direct sensitization appeared to be operative to a different extent with each drug and sequence.

ABBREVIATION: 5-FU = 5-fluorouracil.

[1] Supported by Public Health Service grant CA-37775 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Medical Biophysics Unit, British Columbia Cancer Research Centre, Vancouver, Canada.

[3] We thank Denise McDougal and Nancy Arnold for technical assistance.

* *Reprint requests:* Ralph E. Durand, Ph.D., Medical Biophysics Unit, British Columbia Cancer Research Centre, 601 W. 10th Ave., Vancouver, British Columbia V5Z 1L3, Canada.

MATERIALS AND METHODS

Cell Line and Culture Techniques

Chinese hamster V79-171 lung cells were routinely maintained as monolayers (*7,8*) on plastic petri dishes in Eagle's Minimum Essential Medium plus 10% fetal bovine serum. To initiate spheroid growth, asynchronous cells were removed from the plastic growth dishes by trypsin. Spinner culture flasks (100-ml size) were then inoculated with 10^6 cells in 70 ml of Eagle's Minimum Essential Medium plus 5% fetal bovine serum and were stirred at 180 rpm; the gas phase contained 5% CO_2 in air. During the growth of the spheroids, the medium was first changed on day 3. Spheroids were transferred into 250-ml flasks on day 4 or 5, and spent medium was removed and replaced with fresh medium daily thereafter (*7*).

Drugs and Treatment

Doxorubicin and mitomycin were purchased from Sigma Chemical Co.; clinical formulations of the other drugs were utilized (carmustine and cisplatin prepared by Bristol Laboratories and injectable 5-FU from Hoffmann-La Roche, Inc.). Spheroids 600–750 μm in diameter were pre-equilibrated with an atmosphere of 10% O_2, 5% CO_2 in nitrogen for 30 minutes prior to the initial drug or radiation treatment and were maintained in this atmosphere for exposure to the subsequent agent. Similarly, when drug treatment preceded irradiation by 3 hours, the drug-containing medium was removed, replaced by pre-equilibrated fresh medium, and maintained at the lower oxygen level throughout the incubation period and subsequent irradiation. The number of viable cells per spheroid was again determined before irradiation in this latter protocol to quantify repair of potentially lethal drug damage; repair was not observed for any of the agents investigated here. Radiation was delivered at 4 Gy/minute using a 280-kV (peak) x-ray source; in nonsorting experiments, techniques identical to those previously reported were used (*7*).

Cell-sorting Procedures

Flow cytometry and fluorescence-activated cell sorting utilized a dual-laser Becton Dickinson FACS-440. Spheroids were stained with 2 μM Hoechst 33342 for 20 minutes prior to trypsinization by adding the dye directly to the drug-containing flask for the final few minutes of drug exposure (*9–11*). The stained, disaggregated single-cell suspension was resuspended in at least three volumes of growth medium to one of trypsin and was maintained at 4° C during the sorting procedures (typically $<$1 hr).

For sorting, the primary laser was operated at 400 mW and 488 nm and the forward scatter and 90° scatter signals were monitored; the UV laser was operated at 40-mW power with the 350- to 360-nm lines, and the Hoechst emission was monitored through a bandpass filter (449 ± 10 nm). The cell population was defined on the basis of the forward scatter signal; the resulting signals from the stain and 90° scatter were processed through logarithmic amplifiers, and the ratio of these signals was used to generate a "stain concentration" profile, which was integrated to establish 10 windows of equal cell numbers (*8*). The actual number of cells desired per petri dish and the number of duplicate determinations were preprogramed, and the FACS was essentially utilized as a micromanipulator to deposit *known* numbers of cells into "sort" tubes, which were then poured and rinsed into growth dishes (*12*).

Experiments to assess the toxicity of the Hoechst 33342, alone or combined with radiation or any of the drugs, were negative. However, selective interactions in a subpopulation of cells cannot be identified by measuring the average response of the entire population, and the subpopulations cannot be studied without staining and sorting. Thus, our controls were to use a drug, radiation, or the combination at two to three levels of toxicity, and the stain concentration was then escalated to determine the level at which additional cell killing occurred. Concentrations of Hoechst 33342 at least tenfold higher than those typically used were required to promote additional cell killing.

RESULTS

Drug exposures intended to produce 10% net survival were chosen in all experiments, and the results have been presented in terms of the ability of essentially isoeffective drug exposures to modify cellular response to graded doses of radiation. This design allows survival of adequate numbers of cells after combining the radiation and drug exposures, making it possible to construct response curves with at least four decades of cell kill. The spheroids were equilibrated in a 10% oxygen environment just before drug-radiation exposure, to create essentially equal oxic and hypoxic subpopulations in the spheroid; any differential responses dependent on these factors could then be defined more precisely. Although sorting and conventional assays were not always performed simultaneously in this study, the typical "sensitivity profile" of spheroid cells to each drug exposure, presented as a function of cell location in the spheroid, aided the interpretation of the combined modality response.

It has been reported that doxorubicin interacts with radiation in the clinical (*13,14*) and laboratory settings (*15,16*) and is thus of considerable interest for combined modality studies. Exposure to 8.0 μg/ml for 1 hour resulted in increased differential toxicity to the external cells in the spheroid (fig. 1, *upper panel*). When drug treatment was administered prior to radiation exposure (fig. 1, *lower panel*), a marked overall decrease in cell viability was seen. Little of this potentiation was lost if a 3-hour delay occurred before radiation treatment. When drug exposure followed irradiation, the combined cell killing was *less* than expected from independent action of the modalities (*light curve,* fig. 1, *lower panel*). This reflects the similar resistance patterns of spheroid cells to both agents, though the drug resistance is cell-cycle and penetration dependent (*8*), whereas the radioresistance of the internal cells is due entirely to hypoxia (*10*). Interestingly, these results suggest that this treatment sequence would produce much more toxicity in well-perfused tissues in vivo (normal tissues) than in hypoxic (tumor) cells.

The V79 spheroids are quite resistant to the antimetabolite 5-FU and show little cell-cycle-specific toxicity (*10*); exposure to 500 μg/ml for 2 hours (fig. 2, *upper panel*) produced essentially equal cell killing throughout. This resulted in additive toxicity when irradiation preceded drug exposure (fig. 2, *lower panel*). Preirradiation treatment with 5-FU resulted in a supra-additive response, which was partially reversed if a 3-hour interval was allowed between

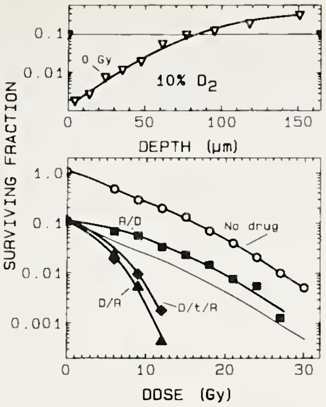


FIGURE 1.—Survival of cells from V79 Chinese hamster spheroids exposed to 8.0 μg of doxorubicin/ml for 1 hr (D) and/or irradiated (R). Toxicity of drug alone vs. cell position in spheroid is shown in *upper panel;* radiation alone (*open circles*) is compared with several combined radiation-drug sequences (*closed symbols*) in *lower panel.* For *curve* D/R, spheroids were irradiated during final minutes of drug exposure; D/t/R indicates drug removal 3 hr prior to irradiation; and R/D indicates that drug exposure immediately followed irradiation. For reference, *horizontal line* in *upper panel* shows average survival of unsorted cells; in *lower panel, light line* represents response to radiation only, normalized to survival level for drug only, thus permitting easier comparison of radiosensitivities of untreated and drug-treated populations.

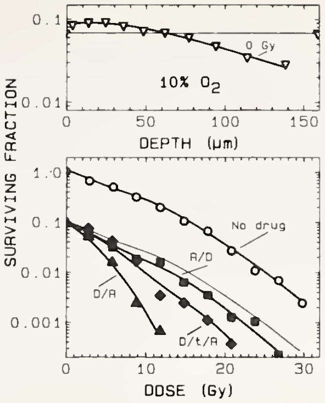


FIGURE 3.—Survival of cells from V79 Chinese hamster spheroids exposed to 2.0 μg of carmustine/ml for 30 min and/or irradiated. Toxicity of drug alone vs. cell position in spheroid is shown in *upper panel;* radiation alone (*open circles*) is compared with several combined radiation-drug sequences (*closed symbols*) in *lower panel.* All other conditions were similar to those for fig. 1. For explanation of *horizontal line* in *upper panel* and *light line* in *lower panel, see* fig. 1 legend.

drug and radiation treatments. The number of cells surviving the drug treatment remains constant during this interval; no potentially lethal damage repair occurs. Thus, 5-FU seems to show both direct (cytotoxic) and indirect (metabolic) effects on the exposed cell populations.

Exposure of spheroids to 2.0 μg of carmustine/ml for 30 minutes resulted in a toxicity profile showing an increased responsiveness of the *internal* cells (fig. 3, *upper panel*). Since these are the cell populations that are most radioresistant, the net effect of either preirradiation or postirradiation drug exposure was enhanced cell kill (fig. 3, *lower panel*), though drug exposure before irradiation was more effective. Partial reversal of these potentiating effects was introduced if a 3-hour interval occurred between treatments.

Mitomycin, an anticancer antibiotic, was of particular interest since it has been reported to show enhanced toxicity in a bioreductive hypoxic environment (*17,18*); we have found its effects to be similarly enhanced in hypoxic V79 cells exposed under monodispersed conditions (data not shown). In spheroids, however, the internal cells are inherently more sensitive to high levels of the drug, but this sensitivity is not further increased by the concomitant hypoxia (*10*). At 0.8 μg/ml for 1 hour, mitomycin was almost equally toxic to both oxic and hypoxic fractions (fig. 4, *upper panel*). In combination with radiation (fig. 4, *lower panel*), the net toxicity was only additive for both preirradiation and postirradiation drug treatments.

Cisplatin has previously been shown to be a radiosensi-

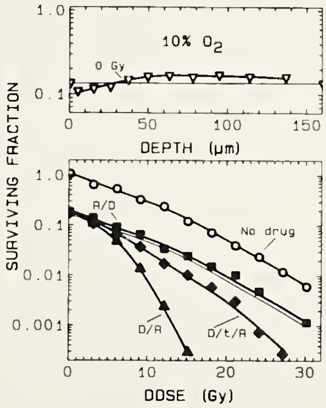


FIGURE 2.—Survival of cells from V79 Chinese hamster spheroids exposed to 0.5 mg of 5-FU/ml for 2 hr and/or irradiated. Toxicity of drug alone vs. cell position in spheroid is shown in *upper panel;* radiation alone (*open circles*) is compared with several combined radiation-drug sequences (*closed symbols*) in *lower panel.* All other conditions were similar to those for fig. 1. For explanation of *horizontal line* in *upper panel* and *light line* in *lower panel, see* fig. 1 legend.

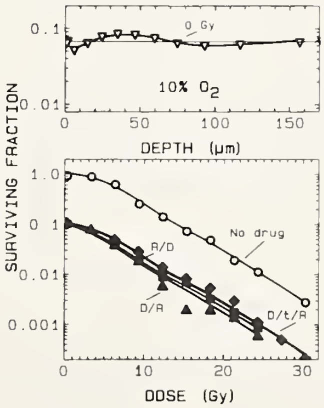


FIGURE 4.—Survival of cells from V79 Chinese hamster spheroids exposed to 0.8 μg of mitomycin/ml for 1 hr and/or irradiated. Toxicity of drug alone vs. cell position in spheroid is shown in *upper panel;* radiation alone (*open circles*) is compared with several combined radiation-drug sequences (*closed symbols*) in *lower panel.* All other conditions were similar to those for fig. 1. For explanation of *horizontal line* in *upper panel, see* fig. 1 legend.

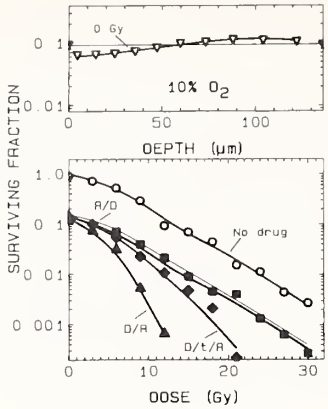


FIGURE 5.—Survival of cells from V79 Chinese hamster spheroids exposed to 7.5 μg of cisplatin/ml for 1 hr and/or irradiated. Toxicity of drug alone vs. cell position in spheroid is shown in *upper panel;* radiation alone (*open circles*) is compared with several combined radiation-drug sequences (*closed symbols*) in *lower panel.* All other conditions were similar to those for fig. 1. For explanation of *horizontal line* in *upper panel* and *light line* in *lower panel, see* fig. 1 legend.

tizer with preferential activity in hypoxic cells (*19,20*). In spheroids, a 1-hour exposure to 7.5 μg of cisplatin/ml resulted in only slight differentials in toxicity as a function of cell position in the spheroid, at the 10% survival level (fig. 5, *upper panel*). The greatest potentiation was seen with the preirradiation sequence; less was observed if a 3-hour delay preceded radiation treatment. With postirradiation drug exposure, the net response was slightly greater than the predicted additive toxicity of the agents given independently.

Even less toxic cisplatin exposure (fig. 6, *upper panel*) clearly increased net cell killing throughout the spheroid when combined with radiation (fig. 6, *lower panel*). The differential toxicity of a 15-Gy dose of radiation, where the external cell populations were more oxic and hence more radiosensitive, was reduced when cisplatin exposure preceded irradiation. The relatively greater downward displacement of the combined modality curves (fig. 6, *lower panel*) indicates that cisplatin *radiosensitizes* all cell populations; the increased toxicity in the internal subpopulations compared to external cells reflects preferential sensitization of those hypoxic cells. At these drug levels, true sensitization is indicated by the fact that the transition from radiosensitivity to radioresistance (aerobic to hypoxic conditions) remained at a constant depth within the spheroids; decreased oxygen utilization would have resulted in a shift of this transition region to a greater depth within the spheroids (*8,21*).

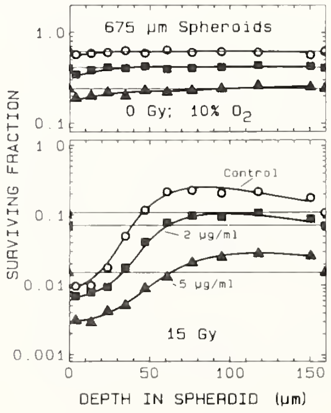


FIGURE 6.—Toxicity of cisplatin alone (*upper panel*) or radiation plus indicated cisplatin exposure for 1 hr (*lower panel*) plotted vs. cell position in spheroid at time of treatment. For *lower panel,* all irradiation was performed during final few minutes of drug exposure; *curves* in *lower panel* show net toxicity, normalized only for plating efficiency of cells in each fraction (*open symbols, upper panel*). Relatively greater downward displacement of curves for drug-treated spheroids in *lower panel* is indicative of radiosensitization even by these relatively nontoxic exposures to cisplatin. For explanation of *horizontal lines* in *upper panel* and *lower panel, see* fig. 1 legend.

TABLE 1.—Drug effect in combination treatment

Drug	Alone[a]	Immediately before irradiation[b]	3 hr before irradiation[b]	Immediately after irradiation[b]
Carmustine	8.0	22.0	14.3	10.6
Cisplatin	8.3	22.5	15.8	8.7
5-FU	9.1	25.6	15.0	7.8
Mitomycin	10.0	12.2	10.0	10.8
Doxorubicin	10.4	26.2	24.2	3.1

[a] Effect of each drug alone is calculated as equivalent No. of grays for identical reduction of survival on terminal exponential region of survival curve for radiation only.

[b] For combined treatment, values shown are reduction in radiation dose needed to produce surviving fractions of 0.001; average doses of 36 and 16 Gy would be required for this level of toxicity under conditions of 10% O_2 as used or after complete reoxygenation, respectively.

To summarize the drug effectiveness in these combination treatments (table 1), the greatest toxicity gain (equivalent to 22–26 Gy) was achieved with drug exposures immediately before irradiation. When a 3-hour interval in the absence of drug was allowed before irradiation, reversal of the radiosensitizing response by the equivalent of about 10 Gy was observed with carmustine, 5-FU, and cisplatin; doxorubicin-treated spheroids showed minimal recovery. Some postirradiation therapeutic gain was observed with carmustine, a result of the drug and radiation cytotoxicity being registered in different subpopulations of cells. The 5-FU, mitomycin, and cisplatin treatments showed postirradiation responses largely predictable on the basis of the drug toxicity alone; doxorubicin was much less toxic (by the equivalent of 7 Gy) to the cells that survived radiation.

DISCUSSION

Combining two or more cytotoxic agents for tumor therapy offers the advantages of the increased response due to additive antitumor effects and also introduces the possibility of interactions between the agents. For the ideal therapeutic response, one of the agents should potentiate the antitumor effects of the other; this supra-additive effect then means that lower dosage and consequently less normal tissue damage are required to achieve the same tumor cell kill. Radiation-drug combinations, when administered to spheroids in various sequences, provide insights into the most effective treatment schedule and also into the perturbations in the spheroid cell populations leading to this net response. Thus, by comparing the responses of the aerobic cycling, aerobic noncycling, and acutely or chronically hypoxic cell subpopulations of the spheroids, it is possible to select combination therapies that preferentially target a defined cell subpopulation, which should increase the therapeutic ratio in the clinic.

Radiation toxicity in V79 spheroids has been well characterized (*7,8*); not surprisingly, cell-cycle and oxygenation status are dominant variables. Drug-induced cytotoxicity has been much less extensively studied, but drugs have been identified with at least some preferential toxicity to internal or external subpopulations of cells (*10*). Consequently, judicious choice of combinations could overcome single-agent resistance, even without requiring any interaction between modalities. Even if a chemotherapeutic agent does not show preferential toxicity for a particular subpopulation, it can still cause an enhanced multimodality response through secondary mechanisms. For example, an agent that suppresses respiration either by overt cytotoxicity or transient metabolic perturbations can cause a decrease in the O_2 consumption of the cells, which in turn induces reoxygenation of the spheroid, increasing cell radiosensitivity and thus the net response (*22,23*). At adequate levels, some chemotherapeutic agents may also act directly as radiosensitizers.

The data presented here indicate that, with the exception of mitomycin, all drugs markedly enhanced cell killing when the drug exposure immediately preceded irradiation. The supra-additive responses of the preirradiation treatments with doxorubicin, 5-FU, carmustine, and cisplatin are all consistent with dual action of the drugs: direct radiosensitization coupled with reoxygenation through direct cytotoxicity and/or metabolic effects. Unlike the results with the other drugs, delaying irradiation after doxorubicin exposure did not significantly reduce the apparent interaction; cellular respiration remained depressed over the 3-hour interval, indicating either rapid cell death or a prolonged metabolic interaction. The 5-FU, cisplatin, and mitomycin exposures showed minimal selective killing of any subpopulation at the concentrations used, and thus, when used after radiation, produced results virtually indistinguishable from independent toxicity. Carmustine was preferentially toxic to the radioresistant internal cells; on the other hand, doxorubicin preferentially killed the external oxic cell population. For postirradiation drug exposures, doxorubicin was thus considerably less effective than carmustine.

Similar though less dramatic results can be deduced from many clinical and animal studies; interpretation of these results is generally more difficult due to the uncertainty in defining the tumor subpopulation most resistant to treatment and due to the end point used: typically, tumor growth delay in experimental systems, which is biased toward the growth fraction of the tumor. Interestingly, positive interactions are generally observed in animal (*20,24,25*) and clinical (*1,2,14,26*) studies only when the drug protocol used is effective as a single agent. Similarly, greater enhancement is often found in multifraction protocols than in single-fraction treatments in animal models (*24,25*), suggesting that subtle effects are induced in each treatment. In contrast, the effects in spheroids of most of the preirradiation drug exposures used here were hardly subtle. Much of the observed potentiation resulted from reoxygenation of the spheroid due to rapid metabolic and/or cytotoxic effects of the drugs. In the spheroid system, unlike animal systems, *effective* drug concentrations can be produced essentially instantaneously and maintained as desired. While adequate drug levels for such obvious secondary effects probably cannot be achieved at the time of irradiation in vivo, even small interactions should be beneficial.

In conclusion, our data indicate that drug-radiation interactions are *not* simple or unique functions of a particular drug. Thus, effective use of drugs with radiation to exploit preferential toxicity, metabolic interactions, and/or direct radiosensitization requires not only appropriate dosages, but also advantageous sequences.

REFERENCES

(*1*) Tubiana M, Arriagada R, Cosset JM: Sequencing of drugs and radiation. The integrated alternating regimen. Cancer 55:2131–2139, 1985.

(*2*) Fu KK: Concurrent radiotherapy and chemotherapy for advanced head and neck cancer. *In* Head and Neck Cancer (Wittes RE, ed). New York: Wiley, 1985, pp 221–248.

(*3*) Inch WR, McCredie JA, Sutherland RM: Growth of nodular carcinomas in rodents compared with multi-cell spheroids in tissue culture. Growth 34:271–282, 1970.

(*4*) Sutherland RM, McCredie JA, Inch WR: Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. J Natl Cancer Inst 46:113–120, 1971.

(*5*) Durand RE: Cell cycle kinetics in an in vitro tumor model. Cell Tissue Kinet 9:403–412, 1976.

(*6*) Olive PL, Leonard JC, Durand RE: Development of tetraploidy in spheroids. In Vitro 18:708–714, 1982.

(*7*) Sutherland RM, Durand RE: Radiation response of multicell spheroids: An in vitro tumour model. Curr Top Radiat Res Q 11:87–139, 1976.

(*8*) Durand RE: Oxygen enhancement ratio in V79 spheroids. Radiat Res 96:322–334, 1983.

(*9*) Durand RE: Use of Hoechst 33342 for cell selection from multicell systems. J Histochem Cytochem 30:117–122, 1982.

(*10*) Durand RE: Chemosensitivity testing in V79 spheroids: Drug delivery and cellular microenvironment. JNCI 77:247–252, 1986.

(*11*) Olive PL, Chaplin DJ, Durand RE: Pharmacokinetics, binding and distribution of Hoechst 33342 in spheroids and murine tumours. Br J Cancer 52:739–746, 1985.

(*12*) Durand RE: Use of a cell sorter for assays of cell clonogenicity. Cancer Res 46:2775–2778, 1986.

(*13*) Poulakos L, Schenken LL, Hagemann RF, et al: Control of local tumor growth with combined fractionated radiotherapeutic and chemotherapeutic regimens. J Natl Cancer Inst 54:1103–1105, 1975.

(*14*) Wang JJ, Cortes E, Sinks LF, et al: Therapeutic effect and toxicity of Adriamycin in patients with neoplastic disease. Cancer 28:837–843, 1971.

(*15*) Durand RE: Adriamycin: A possible indirect radiosensitizer of hypoxic tumor cells. Radiology 119:217–222, 1976.

(*16*) Hellman S, Hannon E: Effects of Adriamycin on the radiation response of murine hematopoietic stem cells. Radiat Res 67:162–167, 1976.

(*17*) Rockwell S, Kennedy KA: Combination therapy with radiation and mitomycin C: Preliminary results with EMT6 tumor cells in vitro and in vivo. Int J Radiat Oncol Biol Phys 5:1673–1676, 1979.

(*18*) Kennedy KA, Teicher BA, Rockwell S, et al: Chemotherapeutic approaches to cell populations of tumors. *In* Molecular Actions and Targets for Cancer Chemotherapeutic Agents (Sartorelli AC, Layo JS, Bertino JR, eds). New York: Academic Press, 1981, pp 85–101.

(*19*) Douple EB, Richmond RC: A review of platinum complex biochemistry suggests a rationale for combined platinum-radiotherapy. Int J Radiat Oncol Biol Phys 5:1335–1339, 1979.

(*20*) Wodinsky I, Swiniarski J, Kensler CJ, et al: Combination radiotherapy and chemotherapy for P388 lymphocytic leukemia in vivo. Cancer Chemother Rep Part 2, vol 4(1):73–97, 1974.

(*21*) Durand RE: Roles of thiols in cellular radiosensitivity. Int J Radiat Oncol Biol Phys 10:1235–1238, 1984.

(*22*) Biaglow JE, Durand RE: The effects of nitrobenzene derivatives on oxygen utilization and radiation response of an in vitro tumor model. Radiat Res 65:529–539, 1976.

(*23*) Durand RE, Biaglow JE: Radiosensitization of hypoxic cells of an in vitro tumor model by respiratory inhibitors. Radiat Res 69:359–366, 1977.

(*24*) Lelieveld P, Scoles MA, Brown JM, et al: The effect of treatment in fractionated schedules with the combination of X-irradiation and six cytotoxic drugs on the RIF-1 tumor and normal mouse skin. Int J Radiat Oncol Biol Phys 11:111–121, 1985.

(*25*) Twentyman PR, Kallman RF, Brown JM: The effect of time between X-irradiation and chemotherapy on the growth of three solid mouse tumors. III. *cis*-Diamminedichloroplatinum. Int J Radiat Oncol Biol Phys 5:1365–1367, 1979.

(*26*) Lichter AS, Bunn PA, Ihde DC, et al: The role of radiation therapy in the treatment of small cell lung cancer. Cancer 55:2163–2175, 1985.

Responses of Tumor Cell Subpopulations to Single Modality and Combined Modality Therapies [1]

Dietmar W. Siemann* and Peter C. Keng [2,3]

ABSTRACT—**By centrifugal elutriation, viable cell subpopulations were isolated directly from KHT sarcomas treated with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, mitomycin (MIT C), cyclophosphamide (CTX), or radiation. Survival in these subpopulations was determined by a clonogenic cell survival assay either immediately (radiation) or 24 hours after treatment with drugs. Following irradiation, late S and G_2/M cells were found to be sensitive, but G_1 cells were preferentially spared. For all chemotherapeutic agents evaluated, survival of cells at the G_1/S boundary 24 hours after drug exposure was at a nadir, whereas cells in the late S and G_2/M at this time were always drug resistant. The response of cells in early G_1 24 hours after drug treatment ranged from sensitive (MIT C) to resistant (CTX). Because of these results, the combination of MIT C and radiation was investigated in detail. When MIT C was administered 24 hours before tumor irradiation, the chemotherapeutic agent significantly reduced the radiation-resistant subpopulations, whereas the radiation treatment effectively killed cells spared by the chemotherapeutic agent. Isobologram analysis further indicated that this treatment combination led to "supra-additive" cell kill in the tumor.—NCI Monogr 6:101–105, 1988.**

Drug action and radiation efficacy can be affected by the complex environmental conditions existing in tumors. For example, tumor cell subpopulations resistant to therapy due to their position in the cell cycle or their location away from blood vessels have long been implicated as major factors influencing the outcome of chemotherapy or radiotherapy (*1–4*). Investigation of the importance of these subpopulations in the overall tumor response to in vivo therapy requires techniques to isolate them directly from the tumors. The development of such techniques has been technically difficult (*5–7*). Nevertheless, direct in situ assessment of anticancer agent activity may be necessary because it has been shown that tumor cells may respond differently to therapy depending on their mode of growth, i.e., in vivo or in vitro (*8–10*). To address this question, we have utilized centrifugal elutriation in conjunction with FCM analysis to evaluate the overall tumor response to therapy in the various subpopulations of cells comprising neoplastic tissues (*7,11–13*). In our present investigations, this method was used to assess the response of KHT sarcomas to radiation and 3 chemotherapeutic agents. On the basis of the single-agent experiments, the in situ sensitivity of the tumor cell subpopulations to combinations of MIT C and radiation was determined in detail. Essential to the evaluation of any combination of agents is a means of analyzing whether the results observed are 1) the additive responses of the individual agents, 2) less than additive, or 3) greater than additive. To address this problem, we used the method described by Steel and Peckham (*14*) of constructing isoeffect plots (isobolograms) to analyze the interaction in KHT sarcomas treated with MIT C prior to irradiation.

MATERIALS AND METHODS

In all experiments, KHT sarcomas (*15*) growing intramuscularly in the hind legs of C3H/HeJ mice were used. Tumors were treated with radiation or various chemotherapeutic agents when they reached a size of 0.6–0.8 g. The chemotherapeutic agents MIT C and CTX were dissolved in saline to yield final concentrations of 1 and 10 mg/ml, respectively. Initially, CCNU was dissolved in absolute ethanol and then, immediately prior to injection, was further diluted with hydroxypropyl methylcellulose to yield a final concentration of 1 mg/ml. All drugs were administered ip according to body weight of the animal. When given in combination, MIT C preceded the radiation treatment by 24 hours.

Tumor irradiations were performed with a cesium-137 source operating at a dose rate of approximately 5.25 Gy/minute. The mice were not anesthetized when they received whole-body irradiation. Twenty-four hours after drug treatment or immediately after tumor irradiation, clonogenic cell survival was assessed with an in vivo to in vitro tumor excision assay (*16*). Briefly, tumors were excised, then dissociated, and the tumor cells were plated in an agar–medium mixture in multiwell plates. Two weeks later, colonies were counted and cell survival was determined (*16*).

Cell populations in various phases of the cell cycle were obtained directly from solid KHT sarcomas by centrifugal elutriation as described (*7,12,13*). Briefly, tumors were dissociated into a single cell suspension, which was then elutriated in ice-cold alpha-minimum essential medium supplemented with 10% fetal calf serum. Approximately 1×10^8 cells were loaded into the separation chamber at a rotor speed of 4,000 rpm and a flow rate of 35 ml/minute.

ABBREVIATIONS: FCM = flow cytometry; MIT C = mitomycin; CTX = cyclophosphamide; CCNU = 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.

[1] Supported by Public Health Service grant CA-36858 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Experimental Therapeutics Division and Department of Radiation Oncology, University of Rochester Cancer Center, NY.

[3] We thank Karen Alliet, Susan Curnick, and Brenda King for technical assistance and Barbara Granger for preparing the manuscript.

* *Reprint requests:* Dietmar W. Siemann, Ph.D., Experimental Therapeutics Division, University of Rochester Cancer Center, 601 Elmwood Ave., Box 704, Rochester, NY 14642.

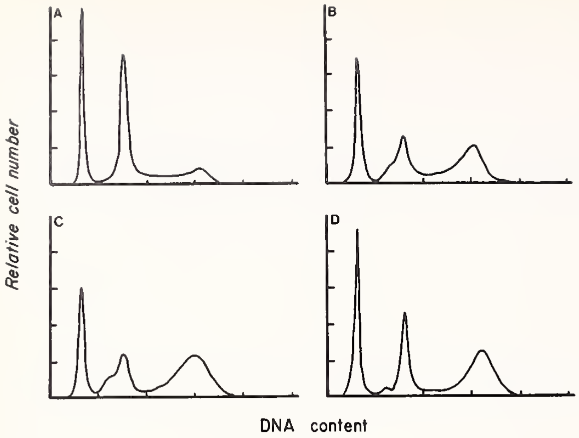


FIGURE 1.—The DNA histograms were obtained by FCM of cells from KHT sarcomas: A) Untreated or immediately after a radiation dose of 15 Gy, B) 6 mg MIT C/kg, C) 50 mg CTX/kg, D) 7.5 mg CCNU/kg. Chemotherapeutic agents were evaluated 24 hr after treatment.

The rotor speed was then decreased in decrements to about 2,000 rpm, with a variable number of 40-ml fractions collected at each interval. The cell number and cell volume distribution for each separated fraction were determined with a Coulter Counter and Channelyzer system. The percentage of cells in each phase of the cell cycle was calculated by FCM for the unseparated cell suspensions as well as for each fraction obtained from the elutriator; cells were stained with mithramycin (*7,13*). Following elutriation, clonogenic cell survival was assessed for every fraction. Because cell suspensions prepared from tumors contain non-neoplastic host cells (*11*), all cell survival values were corrected on the basis of the proportions of host to tumor cells determined from cytospin centrifuge prepared slides stained with Wright's and Giemsa preparations (*11*).

RESULTS

Figure 1 shows the DNA profiles of cells derived from KHT sarcomas treated with various anticancer agents or radiation. Each histogram shows a low fluorescence peak representing diploid non-neoplastic host cells (*7,11–13*), and a bimodal distribution at higher fluorescence intensities representing tumor cells in G_1 and G_2/M, respectively. The proportion of tumor cells in each phase of the cell cycle was determined by computer analysis (*7,12,13*). The DNA histograms indicate a reduction in the proportion of G_1 cells and a substantial accumulation of cells in G_2/M following tumor treatment with each of the chemotherapeutic agents.

Centrifugal elutriation made it possible to separate host from neoplastic cells (*11*) and isolate neoplastic cell fractions with a high degree of cell uniformity (table 1). A similar purity of isolation was achieved for cells separated from drug-perturbed populations as from untreated or irradiated populations (table 1).

For determination of the in situ drug or radiation response across the cell cycle, tumor-bearing mice were killed immediately after irradiation or 24 hours after chemotherapy. The tumors were then dissociated and the cells elutriated. Cell survival, host to neoplastic cell ratios, and DNA content were obtained for each elutriated fraction. The 24-hour interval between MIT C, CCNU, and CTX treatment and cell survival assessment was chosen so as to allow complete expression of chemotherapeutic agent activity and completion of potentially lethal damage repair (*17*). This was not necessary for radiation treatments because KHT sarcomas fail to demonstrate such repair following irradiation (Siemann DW, unpublished data). The survival values for each of the elutriated cell fractions are illustrated in figure 2. Twenty-four hours after chemotherapy, most surviving cells were in the late S and G_2/M phases of the cell cycle, but cells at the G_1/S boundary were particularly sensitive. Cells in early G_1 at this time were relatively resistant to CCNU and CTX but were sensitive to MIT C treatment. In contrast, cells in G_1 were significantly more resistant to radiation than were cells in late S or G_2/M.

Figure 3 shows cell survival across the cell cycle in fractions isolated from KHT sarcomas treated with 6 mg MIT C/kg 24 hours before a 15-Gy dose of radiation. The results indicate that this combination led to uniform cell killing in all cell fractions and suggest that greater than additive killing may have occurred.

The nature of the interaction between MIT C and radiation was studied, and the method of constructing isoeffect plots first to define explicitly the concept of additivity (*14*) was used. This approach requires complete dose–response curves for the agents under investigation, and figure 4 illustrates these for radiation and MIT C. Envelopes of additivity for interactions between MIT C and radiation were constructed at isoeffects of 10^{-3} and 10^{-2} cell survival (fig. 5B) according to the methods of Steel and Peckham (*14*). Then the KHT sarcoma-bearing mice were treated with various doses of MIT C 24 hours before exposure to a range of single doses of radiation (fig. 5A). From these curves, the combinations that resulted in cell survival levels of 10^{-3} or 10^{-2} were determined (e.g., 6 mg MIT C/kg plus 9.5 Gy gives a survival of 10^{-3}). These dose combinations then were plotted on the isobologram (fig. 5B). The results demonstrate that the combinations yielding isoeffects of 10^{-3} or 10^{-2} cell survival all fell below their respective envelopes of additivity. The location of the data points with respect to the envelopes of additivity indicates that combining MIT C with radiation led to a "supra-additive" (*14*) interaction in the tumor.

DISCUSSION

Treatments with anticancer agents often result in cell-cycle, phase-specific cell killing and/or the accumulation of cells in particular phases of the cell cycle (*3*). In the present

TABLE 1.—Purity of elutriated fractions[a]

Treatment	Time of assay, hr	Purity, %		
		G_1	S	G_2/M
Untreated or irradiated	0	90–95	70–75	70–75
6 mg MIT C/kg	24	85–90	75–80	90–95
7.5 mg CCNU/kg	24	90–95	70–75	70–80
50 mg CTX/kg	24	90–95	75–80	90–95

[a] Purity was determined by FCM analysis and computer fitting (*7,13*). Data shown are for the fraction from each phase of the cell cycle that was most homogeneous.

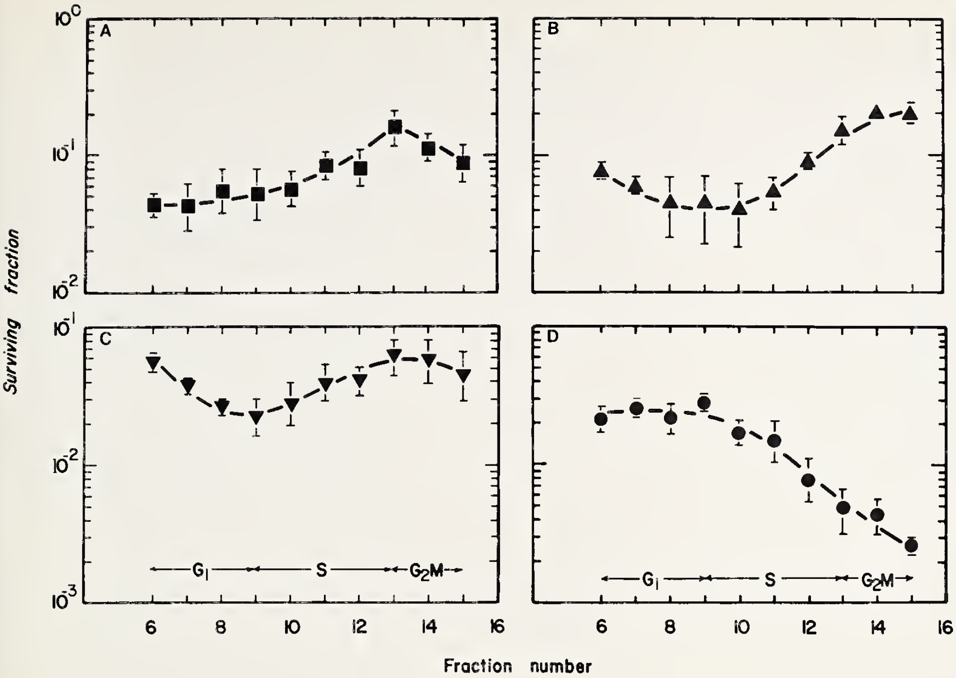


FIGURE 2.—Clonogenic cell survival as a function of cell cycle phase for KHT sarcomas treated in vivo and separated by centrifugal elutriation immediately (radiation) or 24 hr later (chemotherapeutic agents). Results shown are A) 6 mg MIT C/kg; B) 7.5 mg CCNU/kg, C) 50 mg CTX/kg, and D) 15 Gy radiation. Data shown are the mean ± SE of 3–5 experiments.

investigations, the in situ exposure of KHT sarcomas to the alkylating agents (MIT C, CCNU, and CTX) led to significant perturbations in the distribution of cells within the cell cycle as determined by FCM analysis (fig. 1). However, these data alone do not provide information concerning the viability of the treated cells. Yet, from a therapeutic standpoint, it is important that the cell-cycle position of tumor cells which survive the treatment be determined. Centrifugal elutriation in conjunction with FCM analysis allows one to isolate, from treated tumors, cells in the various phases of the cell cycle with the same degree of purity as

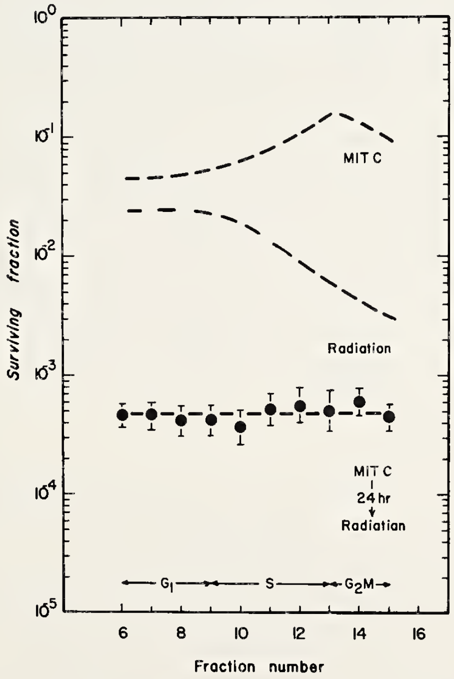


FIGURE 3.—Clonogenic cell survival of KHT sarcoma cells separated by centrifugal elutriation immediately after the completion of an in situ treatment consisting of 6 mg MIT C/kg given 24 hr before a 15-Gy dose of radiation. *Dashed curves* are taken from fig. 2. Results shown are the mean ± SE of 4 experiments.

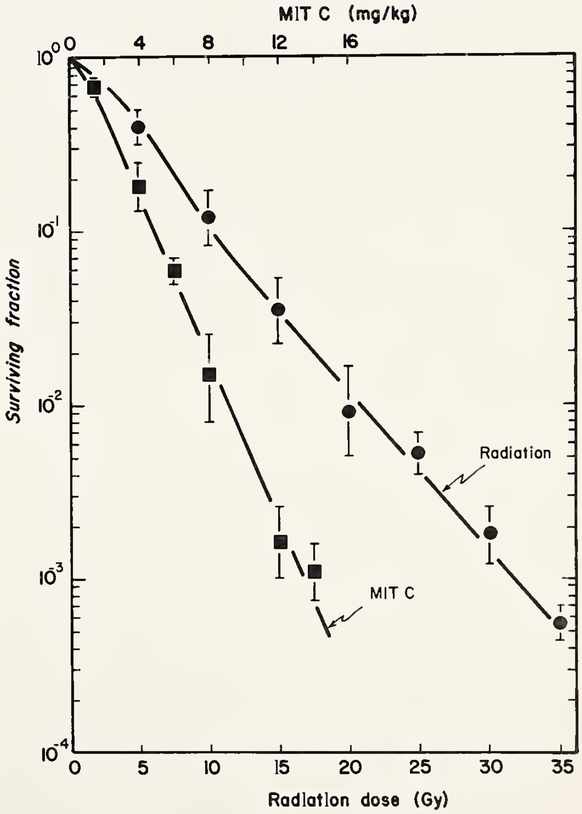


FIGURE 4.—Clonogenic cell survival in KHT sarcomas treated with a range of single doses of radiation or MIT C. Survival was assessed immediately after radiation or 24 hr after drug treatment. Data shown are the mean ± SE of 3–7 experiments.

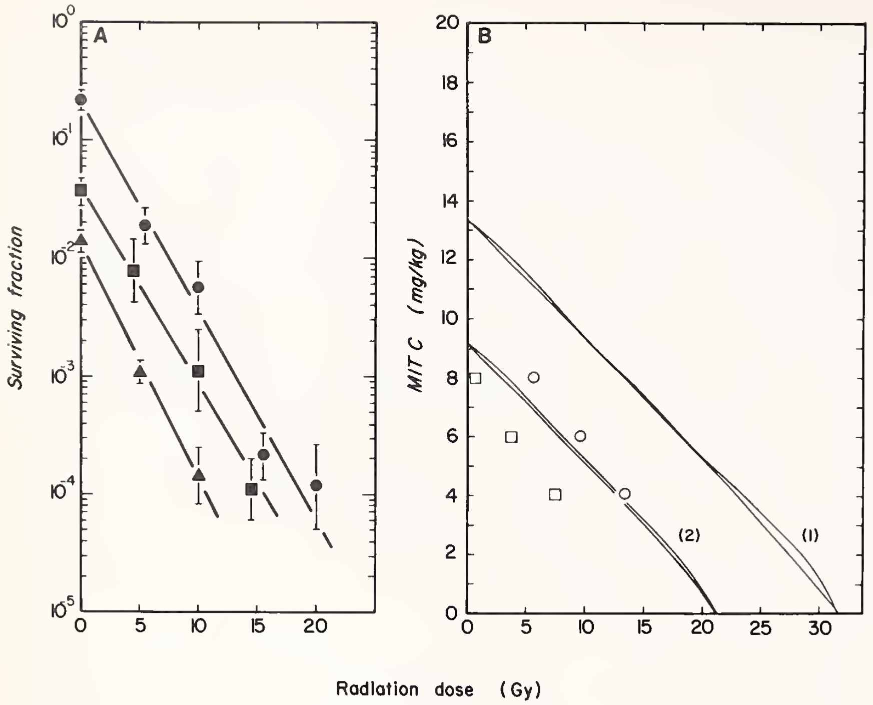


FIGURE 5.—A) Clonogenic cell survival as a function of radiation dose for a range of MIT C doses (●, 4 mg/kg; ■, 6 mg/kg; ▲, 8 mg/kg) given 24 hr before radiation. Points are the mean±SE of 3–4 experiments. B) Envelopes of additivity (curves 1 and 2) constructed from the dose-response curves shown in fig. 4 at isoeffects of 10^{-3} (curve 1) and 10^{-2} (curve 2) tumor cell survival according to the methods of Steel and Peckham (*14*). Data points shown in (B) were determined from (A) at isoeffect survival values of 10^{-3} (○) and 10^{-2} (□).

from untreated tumors (table 1) and to assess their clonogenicity (fig. 2).

When KHT sarcomas were treated with MIT C, CCNU, or CTX, survival 24 hours after treatment was highest in cells found in late S or G_2/M. In contrast, after radiation, cell survival in the G_1 phase predominated (fig. 2D). We (*13*) have shown that in this tumor model radiobiologically hypoxic cells are found primarily in the G_1 phase of the cell cycle. Consequently, the radiation resistance seen in the G_1 cells of the tumor was in part due to the proportion of hypoxic cells in this subpopulation (*13*). Unlike radiation, when KHT tumors were treated with MIT C, cell killing in 24 hours after drug exposure was increased in G_1, and survivors were found preferentially in the late S phase (fig. 2A). Sensitivity in the G_1 phase could reflect the chemotherapeutic agent's previously reported preferential toxicity in hypoxic cells (*18,19*), although some of the surviving G_1 cells at 24 hours may have been in another phase at the time of initial drug exposure.

Because of the survival characteristics of the tumor cell subpopulations following treatment with MIT C and radiation (fig. 2A and 2D), experiments were performed for the determination of whether enhanced tumor responses might be achieved through treatments acting against different tumor cell subpopulations. In the present investigations, MIT C was given 24 hours before tumor irradiation. This interval was chosen so that we could 1) allow sufficient time for full expression of damage and the completion of potentially lethal damage repair, and 2) avoid the complication of MIT C sensitizing the hypoxic tumor cells to radiation. When MIT C and radiation were combined in this manner, the resultant cell survival was found to be uniform throughout all the isolated subpopulations (fig. 3). Therefore, this combination reduced the importance of both hypoxia and cell-cycle specificity in the overall tumor response.

For further study of the nature of the interaction between the two agents, isoeffect plot analysis (*20–22*) was applied to the MIT C–radiation treatment protocol. The tumor response curves for MIT C and radiation shown in figure 4 were used for construction of envelopes of additivity at isoeffect survival levels of 10^{-3} and 10^{-2} (fig. 5B). When MIT C preceded tumor irradiation by 24 hours, the combination data points required to achieve these isoeffects as determined from figure 5A fell well below the envelopes of additivity (fig. 5B). Based on the location of these points within the isobologram, it can be concluded that the MIT C–radiation combination resulted in a supra-additive interaction.

The present results demonstrate that enhanced tumor responses can be achieved by combination treatments acting against different tumor subpopulations. A similar conclusion was reached in our previous investigation of the interaction between CCNU-misonidazole and radiation (*22*). These findings indicate that the use of centrifugal elutriation in combination with FCM analysis and clonogenic cell survival assays may be an effective method for the prediction of which treatment modalities might be used successfully in combination.

REFERENCES

(*1*) BUSH RS, JENKINS RDT, ALLT WEC, et al: Definitive evidence for hypoxic cells influencing cure in cancer therapy. Br J Cancer 37(suppl III):302–306, 1978.

(*2*) GUICHARD M, COURDI A, MALAISE E: Experimental data on the radiobiology of solid tumors. J Eur Radiother 1:171, 1980.

(*3*) TANNOCK I: Cell kinetics and chemotherapy: A critical review. Cancer Treat Rep 62:1117–1133, 1978.

(*4*) SIEMANN DW: Modification of chemotherapy by nitroimidazoles. Int J Radiat Oncol Biol Phys 10:1585–1594, 1984.

(*5*) MEISTRICH ML, GRDINA DJ, MEYN RE, et al: Separation of cells from mouse solid tumors by centrifugal elutriation. Cancer Res 37:4291–4296, 1977.

(*6*) GRDINA DJ, SIGDESTADT CP, PETERS LJ: Phase specific cytotoxicity in vivo of hydroxyurea on murine fibrosarcoma cells synchronized by centrifugal elutriation. Br J Cancer 39:152–158, 1979.

(*7*) KENG PC, WHEELER KT, SIEMANN DW, et al: Direct synchronization of cells from solid tumors by centrifugal elutriation. Exp Cell Res 134:15–22, 1981.

(*8*) HILL RP, NG R, WARREN BF, et al: The effect of intracellular contact on the radiation sensitivity of KHT sarcoma cells. Radiat Res 77:182–192, 1979.

(*9*) MARTIN WMC, McNALLY NJ: The cytotoxic action of Adriamycin on tumour cells in vitro and in vivo. Br J Cancer 41(suppl IV):306, 1980.

(*10*) SIEMANN DW, KOCHANSKI K: Combinations of radiation and misonidazole in a murine lung tumor model. Radiat Res 86:387–397, 1981.

(*11*) SIEMANN DW, LORD EM, KENG PC, et al: Characterization of cell subpopulations dispersed from solid tumours and separated by centrifugal elutriation. Br J Cancer 44: 100–108, 1981.

(*12*) SIEMANN DW, KENG PC: In situ radiation response and oxygen enhancement ratio of KHT sarcoma cells in various phases of the cell cycle. Br J Radiol 57:823–827, 1984.

(*13*) SIEMANN DW, KENG PC: Characterization of radiation resistant hypoxic cell subpopulations in KHT sarcomas. I. Centrifugal elutriation. Br J Cancer 55:33–36, 1987.

(*14*) STEEL GG, PECKHAM MJ: Exploitable mechanisms in combined radiotherapy–chemotherapy: The concept of additivity. Int J Radiat Oncol Biol Phys 5:85–91, 1979.

(*15*) KALLMAN RF, SILINI J, VAN PUTTEN LM: Factors influencing the quantitative estimation of the in vivo survival of cells from solid tumors. J Natl Cancer Inst 39:539–549, 1967.

(*16*) THOMSON JE, RAUTH AM: An in vitro assay to measure the viability of KHT tumor cells not previously exposed to culture conditions. Radiat Res 58:262–276, 1974.

(*17*) SIEMANN DW, MULCAHY RT: Cell survival recovery kinetics in the KHT sarcoma following treatment with five alkylating agents and misonidazole. Int J Radiat Oncol Biol Phys 8:619–622, 1982.

(*18*) ROCKWELL S, KENNEDY KA, SARTORELLI AC: Mitomycin C as a prototype bioreductive alkylating agent: In vitro studies of metabolism and cytotoxicity. Int J Radiat Oncol Biol Phys 8:753–755, 1982.

(*19*) SIEGFRIED JM, SARTORELLI AC, TRITTON TR: Effects of anthracyclines on oxygenated and hypoxic tumor cells. Cancer Res 43:54–59, 1983.

(*20*) DEEN DF, WILLIAMS ME: Isobologram analysis of X ray–BCNU interaction in vitro. Radiat Res 79:483–491, 1979.

(*21*) SIEMANN DW, SUTHERLAND RM: The interaction between Adriamycin and radiation in a solid murine tumor. Radiat Res 83:345–359, 1980.

(*22*) SIEMANN DW, MADDISON K, WOLF K, et al: In vivo interaction between radiation and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea in the absence or presence of misonidazole in mice. Cancer Res 45:198–202, 1985.

Increase in Hypoxic Fraction of Human Colon Tumor Xenografts by Preirradiation of Tumor Bed [1]

John T. Leith [2,*]

ABSTRACT—**In these experiments, the normal tissue in the hip region of nude mice was irradiated with 15 Gy of x-rays. One day after irradiation, 1×10^7 clone A human adenocarcinoma cells were injected to produce solid xenograft tumors. The stromal damage produced by the x-rays yields inhibition of volumetric growth with the time needed to reach 500 mm^3 about 2.2 times that seen in mice bearing tumors in nonirradiated tissue. At a volume of 500–600 mm^3, tumors growing in either irradiated or nonirradiated stroma were either sham irradiated or irradiated with a single large dose of 30 Gy of x-rays. These mice were either breathing air or had been asphyxiated with nitrogen gas (10 min) immediately prior to exposure. Tumors were then excised, enzymatically disaggregated into single cells, and assayed in vitro for clonogenic cell survival. The hypoxic fractions were determined according to the parallel line bioassay technique. The hypoxic fraction in control (non-preirradiated mice) was 3.5%. However, the hypoxic fraction in tumors growing in preirradiated sites was 25.1%. Therefore, the growth of solid tumors in damaged normal tissue may be associated with an increased percentage of hypoxic cells, possibly produced by impairment of vascular supply.—NCI Monogr 6:107–109, 1988.**

We have been investigating the influence of intrinsic cellular heterogeneity of solid tumors on expression of the TBE. The TBE is characterized by changes in volumetric growth that are thought to be primarily due to damage to vascular structure and function (*1–8*). Theoretically, such impairment should favor the production of increased intratumor necrosis (*9*) and, possibly, an increase in the hypoxic fraction. Indeed, Ito et al. (*8*) have shown that the radiocurability of tumors growing in irradiated normal tissue is significantly *less* than that of control tumors, even in the face of a significantly increased tumor regrowth delay. They (*8*) attribute this decreased radiocurability to possible production of an increased fraction of radioresistant hypoxic cells, a result inferred on the basis of experiments on radiation doses that produced cures in 50% of the irradiated tumors, as hypoxic fractions were not directly measured. In this regard, as part of our studies on intraneoplastic diversity, we investigated whether the hypoxic fraction of a pure clonal tumor produced by using the clone A subpopulation obtained from the heterogeneous DLD-1 human colon adenocarcinoma xenograft model system was changed in the TBE situation. Because we have previously shown that these clone A solid tumors have a small hypoxic fraction of about 3% (*10*), we believe that this tumor model might facilitate studies of the potential of an increase in the hypoxic fraction in the TBE situation.

MATERIALS AND METHODS

The DLD-1 tumor system from which the clone A and D subpopulations were obtained has been described in detail (*11*). In these studies, only the clone A tumor line was studied. This cell line is aneuploid and produces poorly differentiated adenocarcinomas when grown in nude mice.

We produced subcutaneous xenografted tumors in nude mice by injecting exponentially growing clone A cells (1×10^7) into the upper hip region in a total volume of 0.25 ml Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, NY). Mice were tagged on the ears for individual monitoring and separated into various groups on a random basis.

Solid tumor volumes were measured by calipers in two orthogonal diameters, and the volumes calculated with the formula for a prolate ellipsoid as we have described (*10*). Volume measurements began at about day 7 postinjection and extended over about the next 50 days. All measurements of tumor volumes were made by a single individual.

For assessment of hypoxic fractions, animals with tumors of approximately equivalent volumes (500–600 mm^3) were used in both the TBE and control situations. For the assays of hypoxic fraction in the TBE-related experiments, mice had previously received 15 Gy of x-irradiation delivered by a Phillips 250-kilovolt peak therapeutic x-ray machine (250 kilovolt peaks, 20 milliamperes, a 50-cm target to surface distance, 1 Gy/min dose rate). Mice were lightly anesthetized by injection of methoxyflurane (Pitman-Moore Inc., Washington Crossing, NJ), restrained with tape, allowed to recover (to minimize blood flow effects due to anesthesia), and locally irradiated with a 4-×6-cm collimator (the trunk of the animal was covered by a 3-mm thick lead shield). Each mouse was individually irradiated while breathing air at ambient temperature. At about 45 days postirradiation, the mice in the TBE studies were separated into 2 groups, and the hypoxic fraction studies were performed. At this time, mice were either sham irradiated or irradiated with a single dose of 30 Gy and were exposed either while breathing air or immediately after asphyxiation with nitrogen gas (10 min). Mice that

---

ABBREVIATION: TBE = tumor bed effect.

[1] Supported by grant No. PDT-243A from the American Cancer Society.

[2] Radiation Biology Laboratories, Brown University, and Department of Radiation Medicine and Biology Research, Rhode Island Hospital, Providence.

[*] *Reprint requests:* John T. Leith, Ph.D., Department of Radiation Medicine and Biology Research, Aldrich-Dexter Bldg., Rhode Island Hospital, 593 Eddy St., Providence, RI 02902.

had not received the 15-Gy TBE conditioning dose were irradiated at equivalent tumor volumes (about 15 days post-injection) and were separated into sham-irradiated and irradiated groups in the same manner as with the TBE experiments. For the 30-Gy treatments, whole-body exposure was performed so that we could process the groups as quickly as possible.

After irradiation, the tumors were excised, minced into small fragments with a scalpel, and enzymatically dissociated into single cells by treatment with 0.5% trypsin (Grand Island Biological Co.) at 37° C for 40 minutes as described (*10*). Tumor cells were counted by hemacytometer and seeded into 60-mm plastic petri dishes (B-D Labware, Rutherford, NJ) containing 5 ml RPMI 1640 medium with 10% fetal bovine serum and 1% penicillin–streptomycin for subsequent assessment of survival according to colony formation. Colonies were allowed to develop for about 14 days and were then fixed and stained with 0.5% crystal violet in absolute methanol. Colonies containing more than 50 cells were counted for subsequent survival analysis. The data were normalized to control values, and the hypoxic fraction was calculated by the relative surviving fractions at 30 Gy (*10*).

RESULTS

The actual volumetric growth curves for tumors growing in either unirradiated or irradiated (15 Gy) mice will be published elsewhere (Leith JT, Bliven SF, Michelson SG, et al, submitted for publication). For the clone A xenografted tumors, we found that the time needed to reach a volume of 500 mm^3 was 19.5 days for the control tumors versus 44.0 days for the mice in the TBE group. These values yield a relative growth rate reduction factor of 2.2, which is similar to other published data for this level of x-irradiation (*7,9,12*). Therefore, a significant TBE exists.

The clonogenic survival data for the mice with tumors in either the control or TBE groups are summarized in table 1. It should be noted that, as these studies were done with comparisons of tumors at equivalent sizes, the colony-forming efficiencies of control tumors versus those in the TBE study are different. However, this decrease in colony-forming efficiencies is a normal event seen in clone A tumors after 40–50 days of growth (*13*) and therefore is not a TBE-associated phenomenon.

TABLE 1.—Colony-forming efficiencies and calculation of hypoxic fractions from excision assay experiments on clone A human colon xenograft tumors

Dose to normal tissue bed, Gy	Dose to tumor, Gy	Clonogenic cell survival, %		Hypoxic fraction[a]
		Oxic tumors	Hypoxic tumors	
0	0	23.30 (4.2)[b]	19.5 (7.3)	
0	30	0.022 (0.011)	0.51 (0.13)	0.036 (0.025)[c]
15	0	5.72 (1.61)	6.41 (2.22)	
15	30	0.038 (0.008)	0.17 (0.008)	0.251 (0.106)

[a] We calculated the hypoxic fraction by using the relationship: HF = [(oxic survival at 30 Gy)/(oxic survival at 0 Gy)]/[(hypoxic survival at 30 Gy)/(hypoxic survival at 0 Gy)].

[b] *Values in parentheses* are the SEM values.

[c] The SE on the estimates of the hypoxic fractions have been propagated and include the error values from the control groups.

We have previously shown that the parallel line bioassay for calculation of the hypoxic fraction is valid for this tumor system (*10*). Using the relative survival values found at the 30-Gy dose level, we wish to make several points. First, the calculation of the hypoxic fraction in the control tumors, a value of 3.6%, agrees well with our previous data (*10*). Second, although the survival found for tumor cells disaggregated from tumors from the TBE mice irradiated in the *hypoxic* condition is statistically identical to previously published values (*10*) and to the data for the control hypoxic tumors presented in this communication, there is a clear increase in the survival value obtained from the tumors from the TBE mice breathing air. This increase yields an apparent hypoxic fraction value of 25.1%, a value that is significantly higher (*t*-test, $P<.05$) than that in the control group (*14*).

DISCUSSION

As previously stated, this work was undertaken as part of a larger study during which we investigated the influence of intrinsic tumor heterogeneity on the expression of the TBE. However, we were also able to perform these associated studies that were specifically intended to investigate the conjecture of Ito et al. (*8*) on the possibility of an increased hypoxic fraction in tumors growing in damaged normal tissue stroma.

The outcome of these experiments has provided a positive answer, at least for this model tumor system. We have obtained data that are statistically significant and indicate a substantial increase in the hypoxic fraction of tumors growing in a TBE situation. The hypoxic fraction has increased from about 4% to about 25%, an increase of 500%. To our knowledge, this is the first time that such an effect has been described when the parallel line bioassay approach was used for estimation of the hypoxic fraction.

The importance of this observation is self-evident. An increased hypoxic fraction in recurrent tumors regrowing in a previously irradiated normal tissue would produce a situation in which radiotherapy re-treatment would be ineffective, particularly as the tolerance of the surrounding normal tissue would already be compromised. Milas et al. (*7*) have demonstrated that a TBE can be seen from fractionated protocols similar to conventional radiotherapy practice as well as from large single-dose irradiation; this would suggest that the possibility of an altered hypoxic state would also result from clinical radiotherapy practice. This result may not be of critical importance to radiotherapy, as previously treated fields would rarely be re-treated. An exception to this might be cranial irradiation in patients who had previously received prophylactic irradiation from the viewpoint of the central nervous system as a sanctuary site. Still the observation of an increased hypoxic fraction in the TBE situation would also likely imply impaired vascular function, which would be associated with a deficit in the effectiveness of the use of chemotherapy for either in-field recurrences or radiation-induced secondary neoplasms. Further support for the concept that recurrent tumors might present a serious clinical challenge arises from the studies of Rice et al. (*15*) in which hypoxic stress of cells during the S phase led to DNA overreplication, gene amplification, and methotrexate resistance.

REFERENCES

(*1*) Hewitt H, Blake E: The growth of transplanted murine tumours in preirradiated sites. Br J Cancer 12:808–824, 1968.

(*2*) Summers W, Clifton K, Vermund H: X-Irradiation of the tumor bed. I. A study of the indirect actions of radiation on transplantable tumors. Radiology 82:691–703, 1964.

(*3*) Jirtle R, Rankin J, Clifton K: Effect of X-irradiation of tumour bed on tumour blood flow and vascular response to drugs. Br J Cancer 37:1033–1038, 1978.

(*4*) Urano M, Suit H: Experimental evaluation of tumor bed effect of C3H mouse mammary carcinoma and C3H fibrosarcoma. Radiat Res 45:41–49, 1970.

(*5*) Trott K, Kummemehr J: Split dose recovery of a mouse tumour and its stroma during fractionated irradiation. Br J Radiol 55:841–846, 1983.

(*6*) Begg A, Terry N: Modification of stromal radiosensitivity by misonidazole and WR-2721. Br J Radiol 50:565–570, 1983.

(*7*) Milas L, Ito H, Hunter N, et al: Retardation of tumor growth in mice caused by radiation-induced injury to tumor bed stroma: Dependency on tumor type. Cancer Res 46:723–727, 1986.

(*8*) Ito H, Barkley T, Peters L, et al: Modification of tumor response to cyclophosphamide and irradiation by preirradiation of the tumor bed: Prolonged growth delay but reduced curability. Int J Radiat Oncol Biol Phys 11: 547–553, 1985.

(*9*) Camplejohn R, Penhaligon M: The tumour bed effect: A cell kinetic and histological investigation of tumours growing in irradiated mouse skin. Br J Radiol 58:443–451, 1985.

(*10*) Leith J, Bliven S, Lee E, et al: Intrinsic and extrinsic heterogeneity in the responses of parent and clonal human colon carcinoma xenografts to photon irradiation. Cancer Res 44:3757–3762, 1984.

(*11*) Dexter D, Spremulli E, Fligiel Z, et al: Heterogeneity of cancer cells from a single human carcinoma. Am J Med 71:949–956, 1981.

(*12*) Begg A, Denekamp J: Stromal damage as a complication in the interpretation of tumour growth delay. Eur J Cancer Clin Oncol 19:1639–1643, 1983.

(*13*) Leith JT, Michelson S, Faulkner LE, et al: Growth properties of artificial heterogeneous human colon tumors. Cancer Res 47:1045–1051, 1987.

(*14*) Goldstein A: Biostatistics. New York: Macmillan, 1964.

(*15*) Rice G, Hoy C, Schimke R: Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. Proc Natl Acad Sci USA 83:5978–5982, 1986.

Development and Application of a Rat Tumor Model for Human Bronchial Carcinoma[1]

H. B. Kal,[2,*] A. H. van Berkel,[2] B. van der Vecht-de Jong,[2] D. W. van Bekkum,[2] and C. Zurcher[3,4]

ABSTRACT—Five squamous cell carcinomas were induced in the lungs of WAG/Rij rats by radiation emitted from isotopes iridium-192 or iodine-125. Tumor fragments were transplanted subcutaneously in syngeneic hosts for propagation of the tumors. A lung cancer model based on implantation of tumor fragments in the lung has been developed. Tumor implants in the lung grew into large invasive squamous cell carcinomas. Metastases in the renal cortex were frequently observed. Tumor growth was determined from repeated chest radiographs. Volume changes after cytostatic treatment could be monitored accurately up to several months. Squamous cell carcinomas transplanted subcutaneously responded as heterogeneously to a variety of cytostatic drugs as did their human counterparts. Responses of the tumor line L17 to doxorubicin were similar when tumors were growing intrapulmonarily or subcutaneously. However, the response of L33 tumors to cisplatin was different, depending on the location. The tumors growing in the lungs provide a model for realistic testing of regimens involving radiation doses, cytostatic drugs, and combinations thereof.—NCI Monogr 6:111–114, 1988.

Lung cancer is the leading cause of death in male cancer patients and the second leading one in females (*1*). Based on current trends, the expectation is that it will soon become the most frequent cause of cancer mortality among women as well.

The distribution of the major histologic types of lung cancer according to the World Health Organization classification is squamous cell carcinoma (40%), adenocarcinoma (10%), large cell carcinoma (20%), and small cell carcinoma (20%). The non-small cell carcinomas are relatively resistant to chemotherapy compared with small cell lung carcinomas. Response rates to various combinations of chemotherapy regimens in patients with non-small cell carcinomas varied between 20% and 40% without evidence of prolongation of survival (*2*).

The cure rate of bronchial carcinomas has not shown much improvement during the past decennia. Clearly, more detailed information on the specific biologic properties of the various forms of bronchial cancer is needed before a more rational approach to the improvement of treatment can be made.

In the past few years, a program was initiated for development of realistic animal models for human bronchial cancer. With tumors induced in the rat lung, we developed a method to implant tumor fragments into the lung. These tumor implants grew to carcinomas resembling primary tumors in man. The development of the model, the growth characteristics of tumors growing intrapulmonarily or subcutaneously in the flank of the rat as well as responses to chemotherapeutic drugs and radiation will be reported.

MATERIALS AND METHODS

Specific-pathogen-free derived female WAG/Rij inbred rats 3 months old and weighing 130 to 160 g were used. Five transplantable, moderately to well-differentiated squamous cell carcinomas were induced in the lungs of WAG/Rij rats by means of iridium-192 wires or iodine-125 seeds implanted intrapulmonarily as described earlier (*3*). In short, 40 male rats received iridium-192 implants with an activity of 17.4 megabecquerel. Twenty female rats received implants of iodine-125 seeds 4.5 mm long and 0.8 mm in diameter, with an activity of 14.8 megabecquerel. In a 14-month observation period, 75% of the animals with implants of iridium-192 wires developed tumors, among which malignant hemangioendotheliomas occurred with the highest frequency (50%). Four transplantable squamous cell carcinomas could be derived from the observed tumors. In 17 months of observation, 3 rats with implants of iodine-125 seeds developed tumors, among which 1 squamous cell carcinoma could be identified. Tumor fragments of the 5 squamous cell carcinomas coded L17, L33, L37, L41, and L42 were transplanted in syngeneic hosts for propagation.

The method of implantation of tumor fragments into the lung is as follows: Animals were anesthetized ip with 200 mg 2,2,2-tribromoethanol/kg. A lateral incision of about 1.5 cm was made between the 7th and 8th ribs. The posterior lobe of the right lung was brought outside the thoracic cavity with a forceps. In the meantime, a tumor fragment was loaded in a trocar with an inner diameter of 1.3 mm and an outer diameter of 1.6 mm. The tumor fragment was implanted in the lobe. In several instances when breathing stopped, we applied forced inhalation using a hand-operated pump.

After implantation of the tumor fragment, the wound in the lobe was coagulated and the lobe was placed back into the thoracic cavity. The incision was closed with a few stitches, and the overlying skin was closed with several wound clips, after which the treated area was sprayed with

---

ABBREVIATIONS: SGD = specific growth delay(s); TD = tumor volume doubling time(s).

[1] Supported by a grant from the Koningin Wilhelmina Fonds, The Netherlands Cancer Foundation.

[2] Radiobiological Institute TNO, Rijswijk, The Netherlands.

[3] Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands.

[4] We thank Mr. H. Goedoen for technical assistance.

* *Reprint requests:* H. B. Kal, Ph.D., Radiobiological Institute TNO, Lange Kleiweg 151, 2288 GJ Rijswijk, The Netherlands.

Nobecutan. A more detailed description of the implantation technique has been published recently (*4*).

About 6 animals can be provided with intrapulmonary tumor implants in 1 hour by 2 skilled technicians. Only 1 rat of a group of 100 rats died perioperatively. With the standardized technique, "take" rates varied between 90% and 100%.

Starting 3 to 5 weeks after implantation, animals were inspected for the presence of tumors by chest radiographs in the ventrodorsal and lateral directions. Radiographs were made while the animals were kept in an upright position and immobilized with an im injection of 1 ml Hypnorm/kg. Radiographs were taken once a week by technicians using a diagnostic Röntgen apparatus, CGR Senographe 1, operating at 30 kV.

Diameters of the lung implants were measured from the radiographs in three perpendicular directions. We calculated tumor volumes from the tumor diameters, a, b, and c, using the formula: $V = \pi abc/6$.

Volume changes of tumors growing subcutaneously in the flanks of WAG/Rij rats were derived from measurements of three tumor dimensions with vernier calipers or from radiographs in two dimensions with the formula: $V = \pi ab^2/6$, in which b is the smaller diameter.

For the determination of growth characteristics of the lung tumors, animals were killed with an overdose of pentobarbital at various times after implantation. A complete necropsy was performed. The lungs were fixed by intratracheal insufflation of 10% buffered formalin. Other tissues which showed abnormalities at necropsy were fixed by infusion in 10% buffered formalin. Tissue samples were routinely processed to 3-μm slides, stained with hematoxylin phloxine saffron, and examined histologically.

Local irradiation of transplantable tumors growing subcutaneously was performed with 300-kilovolt x-rays from a Philips Müller x-ray generator operated at 10 milliamperes and a dose rate of 3.4 $\text{Gy} \cdot \text{min}^{-1}$. Tumors were treated with a variety of chemotherapeutic drugs. The drugs used were methotrexate (3×10 mg/kg, every 3.5 hr, ip), mitomycin (1.5 mg/kg, iv), doxorubicin (7.5 mg/kg, iv), vinblastine (1.5 mg/kg, ip), and cisplatin (6 mg/kg, iv).

Tumor volume growth delay in treated tumors is defined as the time required to reach a volume equal to that at the time of treatment or the time required to reach a volume twice as great as that at the time of treatment, with subtraction of the value for the volume doubling time of the untreated tumor. The latter is applied when a volume reduction after treatment was not observed. For intercomparison of the responsiveness of the different tumors, the method of SGD was used. The SGD is the tumor volume growth delay divided by the TD of the untreated control tumor.

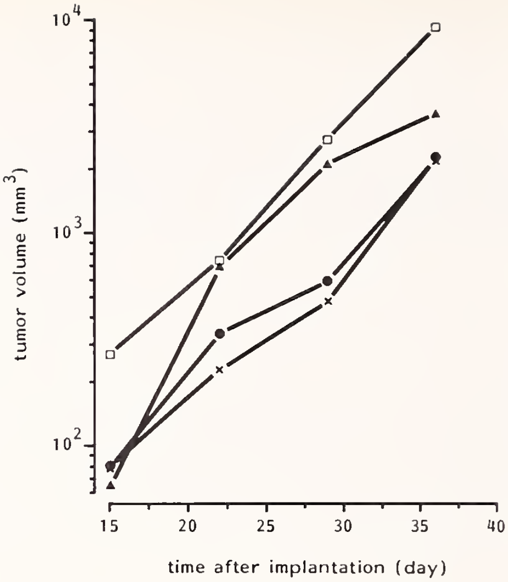


FIGURE 1.—Growth curves derived from radiographs of individual L17 tumors growing subcutaneously.

RESULTS

Tumors growing subcutaneously in the flank of WAG/Rij rats showed a different growth pattern from those developing in the lung. This is shown by a difference in TD (table 1). In general, the doubling times of intrapulmonary tumors are larger than those of tumors growing in the flank for tumor volumes in excess of 400 mm^3. Examples of growth patterns of the L17 tumor are shown in figures 1 and 2.

All squamous cell carcinoma lines showed a capacity to metastasize. The lung tumors disseminated to the mediastinum, lung, kidney, and subrenal area. Tumors of at least two lines growing subcutaneously disseminated to the mediastinum, lungs, and kidneys (table 2). The frequencies with which the tumors disseminated are also indicated.

The histologic growth characteristics of the 5 squamous cell carcinoma lines were investigated. All implants in the lung resulted in highly malignant carcinomas invading the surrounding lung parenchyma, bronchi, and vessels. About 50% of the tumor implants invaded the pleura. All implants except those of the L37 tumor line caused compression of

TABLE 1.—Histologic appearance and TD of squamous cell carcinomas

	Subcutaneous		Intrapulmonary	
Tumor line	Histology	TD, day ($n=5$)	Histology	TD, day[a] ($n=5$)
L17	Well differentiated	4.7±0.7	Well differentiated	9.6±1.5
L33	Well differentiated	5.6±0.6	Well differentiated	9.7±0.5
L37	Poorly to moderately differentiated	5.9±0.5	Moderately to well differentiated	8.9±1.9
L41	Well differentiated	5.8±0.9	Well differentiated	10.9±1.0
L42	Well differentiated	4.1±0.7	Well differentiated	12.4±1.6

[a] Tumor volumes were greater than 400 mm^3.

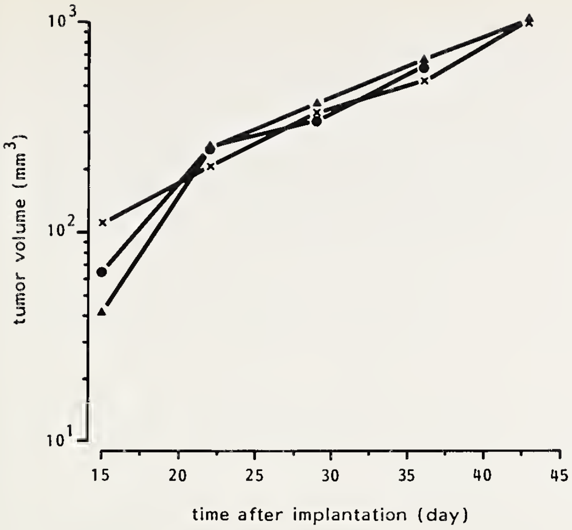


FIGURE 2.—Growth curves derived from radiographs of individual L17 tumors growing intrapulmonarily.

the surrounding lung tissue. Those of the L37 tumor line caused compression in only about 25% of the rats. In contrast to the other cell lines, L37 was less differentiated in the direction of squamous cells and was characterized by less necrosis and more frequent pleural metastasis.

Subcutaneous implants developed into large cystic masses (>1 cm^3), consisting of large central areas of necrosis with keratinaceous debris surrounded by a rim of viable squamous cell carcinoma cells infiltrating into the surrounding connective tissue. Ulceration of overlying skin was frequent in L41 tumors.

We treated the subcutaneous tumors with 300-kilovolt x-rays to determine their radiosensitivity; the SGD are shown in table 3. Also shown are SGD induced by chemotherapeutic drugs.

TABLE 4.—Responses of L17 squamous cell carcinomas after iv treatment with 7.5 mg doxorubicin/kg

Implantation site	Tumor growth delay, days	Tumor cure rate
Subcutaneous	24; 25 56; 65	4/8
Intrapulmonary	24 71	3/5

Two tumor lines, the L17 and L33, were selected for comparison of their responsiveness to drugs when implants were grown subcutaneously or intrapulmonarily. We wished to determine whether the tumor bed may play a role in the response to treatment.

Doxorubicin was administered to 5 rats bearing the L17 lung tumor and to 4 with 8 tumors growing in both flanks at volumes of about 300 to 400 mm^3. The TD of untreated tumors in the lungs and flanks were 9.6 and 4.7 days, respectively. All tumors in both sites showed volume reduction after treatment. Histologic examinations of those tumors still in regression at 3 months after treatment revealed no viable tumor material. The growth delays observed and the tumor cure rates are shown in table 4. The condition of the animals at the end of the 3-month period was poor because of severe kidney damage due to the high dose of doxorubicin.

Responses of L33 tumors in the flanks or lungs were determined after treatment with cisplatin, which, in flank tumors, caused a tumor growth delay of 5.6 ± 0.5 days ($n=6$). Growth of intrapulmonary L33 tumors after cisplatin treatment was not significantly different from that of control tumors; the tumor growth delay was -0.9 ± 2.7 days ($n=5$).

DISCUSSION

We initiated the induction program to establish tumor models of human bronchial cancer representing the most

TABLE 2.—Metastasizing capacity of rat primary squamous cell carcinomas growing intrapulmonarily or subcutaneously

	Subcutaneous tumor metastasis in:			Intrapulmonary tumor metastasis in:			
Tumor line	Lung	Mediastinum	Kidney	Lung	Mediastinum	Kidney	Subrenal area
L17	2/24	0/24	1/24	7/40	28/40	20/40	2/18
L33	0/15	0/15	0/15	0/38	21/38	10/20	—
L37	20/32	13/32	2/32	9/56	54/56	14/41	4/34
L41	0/3	0/3	0/3	2/16	11/16	1/16	1/6
L42	0/3	0/3	0/3	0/10	2/10	1/10	—

TABLE 3.—Responsiveness of squamous cell carcinomas growing subcutaneously to a single dose of radiation and doses of chemotherapeutic drugs[a]

	SGD					
Tumor line	20 Gy x-rays	Vinblastine, 1.5 mg/kg	Mitomycin, 1.5 mg/kg	Methotrexate, 3×10 mg/kg	Cisplatin, 6 mg/kg	Doxorubicin, 7.5 mg/kg
L17	3	0.7	0.1	3.7	0.3	12
L33	1.5	0	0.5	0	1	1.2
L37	3.2	0	0.2	0	0.6	1.0
L41	5	0.4	0.4	0.4	0.8	1.7
L42	ND	0.7	6.3	0.9	5.9	1.4

[a] The SEM is about 20% of the values quoted. ND=not done.

common histologic types in man. So far, only squamous cell carcinomas have been induced and established as transplantable tumors. This observation is similar to the findings of van Bekkum (personal communication) and of Gracey and co-workers (*5*) that local irradiation of lung tissue is an efficient means of inducing malignant lung tumors and yields a relatively large number of squamous cell carcinomas.

The implantation method as applied here is different from that described by Byhardt et al. (*6*), in which intrabronchial installation of a tumor cell suspension was used to obtain intrapulmonary tumor growth. They were able to monitor tumor volume changes by means of x-rays up to only a few weeks after radiographic detection. In our experiments, volume changes can be monitored very accurately up to several months. In addition, the take rate in our tumor lines is larger than the 65% reported by Byhardt et al. Furthermore, multifocal growth was common in their tumor system, whereas in our present study, growth at a single localization is predominant.

With all 5 squamous cell lines, distant metastases were observed in variable frequencies. The majority of the metastases were observed when the primary tumors were relatively large (>500 mm^3). Intrapulmonary growth of all tumor lines disseminated to the mediastinum, lungs, kidneys, and subrenal area. Subcutaneous L17 and L37 tumors disseminated to the lungs, mediastinum, and kidneys. Based on the distribution frequencies, the dissemination pattern of the subcutaneous tumors probably is to the lungs and from there to the mediastinum and kidneys. The dissemination pattern of intrapulmonary tumors is directly from the tumor to the kidneys, adrenals, lungs, and mediastinum.

The growth characteristics of implants of the 5 tumor lines in the lung have common features with respect to invasiveness into the alveoli, bronchi, vessels, and pleura. Only the L37 tumor line showed a different histologic appearance and a more locally aggressive behavior. The high frequency of metastases in the mediastinum because of cells released after tumor growth into the pleural surface may be the result.

The growth characteristics of subcutaneous implants are quite similar. Only the L41 tumor frequently invaded the overlying skin, making this tumor line unsuitable for growth delay studies on subcutaneous development. The TD of flank tumors were smaller than those of the lung with volumes in excess of 400 mm^3. For smaller tumor implants growing intrapulmonarily, smaller TD can be derived that are comparable to those of subcutaneous implants (*4*).

Although the carcinomas we studied are all of the same histologic type, the responses of implants in the flank to a single dose of radiation and to treatments with chemotherapeutic drugs were as heterogeneous as their human counterparts, varying from no response at all to prolonged growth delays. It must be noted that in this study only single doses were used except for methotrexate and that responses to more fractions may yield other results.

From the data obtained, it was determined that all tumor lines responded to radiation, with SGD of more than 1.5 after a single dose of 20 Gy. When the SGD is less than 1, i.e., the growth delay is less than 1 TD, the tumor line can be considered as being insensitive to the treatment. Only the L17 tumor line is sensitive to methotrexate as is the L42 line to mitomycin. All lines are sensitive to treatments with doxorubicin, among which the L17 tumor line is very sensitive. In this line, even some cures were obtained. It must be noted that the dose of doxorubicin is high (7.5 mg/kg) and, in the kidneys, causes severe tubulus dilatation and atrophy with cast formation and interstitial fibroses 3 months after treatment. Not one of the tumor lines was sensitive to treatment with vinblastine, and only two (L33, L42) were sensitive to cisplatin. However, when the dose of the latter drug was increased to 10 mg/kg, SGD of 1.4 and larger were observed for the other lines as well. This drug dose caused late damage in the kidneys; a dose of 6 mg/kg can barely be tolerated.

Only a few experiments have been performed in which the responsiveness of tumors growing intrapulmonarily has been recorded. So far, only responses of the tumor lines L17 and L33 have been collected. Implants of the L17 line in the lung showed the same responsiveness to doxorubicin compared with their counterparts in the flanks. The L33 tumors growing intrapulmonarily did not respond to a treatment with 6 mg cisplatin/kg, whereas the subcutaneous tumors had growth delays of about 1 TD. Whether the cisplatin uptake in the intrapulmonary growths is less than in subcutaneous tumors has to be established to explain the difference observed; in the latter tumors, the response to the drug was dose dependent, as already indicated.

Comparison of the results we obtained with flank and lung tumors will be important for determining in more detail the role of the tumor bed in response to treatments. These lung tumors provide a model with which we can test realistically regimens involving radiation doses, cytostatic drugs, and combinations thereof. The metastatic capacity of the tumor lines will be exploited to develop a minimal residual disease model, which will enable us to evaluate responses of small metastases to supralethal drug doses in combination with autologous bone marrow rescue as a possible treatment modality for human non-small cell carcinomas.

REFERENCES

(*1*) Silverberg E: Cancer statistics, 1984. CA 34:7–23, 1984.

(*2*) Aisner J, Hansen HH: Commentary: Current status of chemotherapy for non-small cell lung cancer. Cancer Treat Rep 65:979–986, 1981.

(*3*) Kal HB, Zurcher C, van Bekkum DW: Induction of lung tumors by radioactive isotopes implanted in the rat lung. JNCI 76:943–946, 1986.

(*4*) Kal HB, van Berkel AH, Zurcher C, et al: A rat lung cancer model based on intrapulmonary implantation of tumour material. Radiother Oncol 6:231–238, 1986.

(*5*) Gracey DR, Fish JE, Divertie MB: Experimental squamous cell lung tumors in Sprague-Dawley and murine pneumonitis-free rats. Cancer 44:598–603, 1979.

(*6*) Byhardt RW, Almagro UA, Fish BL, et al: Development of a rat lung cancer model. Int J Radiat Oncol Biol Phys 10:2125–2130, 1984.

Effect of Spirogermanium and Radiation Therapy on the 9L Rat Brain Tumor Model

Bruce F. Kimler,[1,*] Douglas F. Martin,[1,2] Richard G. Evans,[1] Robert A. Morantz,[2] and Tribhawan S. Vats[1,3]

ABSTRACT—Spirogermanium (SPG) was investigated in the 9L rat brain tumor model in vivo and in vitro. Used at a single ip dose of 50 or 60 mg/kg or at 5 daily doses of 10 mg/kg, SPG was ineffective in prolonging survival of rats burdened with the intracerebrally implanted tumor, i.e., the median survival time (MST) was the same as that for the controls. Only a schedule of 3×20 mg SPG/kg every other day improved the MST compared with controls. Single-dose (20-Gy) radiation therapy (RT, cesium-137 whole-head irradiation) did prolong survival. However, when single-dose SPG was combined with RT (1 hr or 1 day before, or 1 hr after RT), the survival response was worse than after RT alone. When the daily SPG was combined with daily RT (5 doses of 6 Gy), survival was no better than after daily RT alone. In vitro, SPG produces a concentration-dependent, exponential decrease in cell survival as measured by colony formation assay. When combined with radiation, there is an additive effect on cell lethality. Aside from the possibility that SPG does not penetrate the rat brain tumor itself, we have no explanation why SPG shows some activity against human brain tumors and is cytotoxic against 9L cells in vitro, yet is both ineffective by itself and fails to potentiate RT in the 9L rat brain tumor model.—NCI Monogr 6:115-118, 1988.

Spirogermanium (NSC-192965) is an azaspirane compound that is unusual in that a carbon atom has been replaced with a germanium atom in the spirane structure (*1*). In preclinical animal studies (*2,3*) and clinical phase I testing (*4*), SPG demonstrated a lack of hematopoietic toxicity and/or myelosupression. This led to a number of phase II clinical trials (*5*). To date, the most serious toxicities with SPG have been those of a central nervous system nature: dizziness, ataxia, and seizures (*4,5*). Although this dose-limiting neurotoxicity is a problem in most studies, it provides a rationale for testing this agent against primary tumors of central nervous system origin. That is, it provides clinical evidence that SPG can adequately cross the blood–brain barrier. The failure to cross the blood–brain barrier is a problem that limits efficacy of many new agents that might otherwise be active against primary brain tumors. On this basis, a Southwest Oncology Group phase II study was conducted in which the efficacy of SPG in central nervous system tumors was examined (*6*). Goodwin et al. found that SPG did show modest activity in a population of heavily pretreated patients, and it has been recommended for further trials in combination with other agents.

With the above background, we decided to study this promising chemotherapeutic agent in the 9L rat brain tumor model (*7*). The documented therapeutic efficacy of such agents as RT (*8*), carmustine (*9*), and bleomycin (*10*), alone or in various combinations (*11–14*), against the 9L rat brain tumor is consistent with the observed effectiveness of these agents against human brain tumors. This communication reports the investigation of the therapeutic effects in the 9L rat brain tumor of SPG, used alone and in combination with RT.

MATERIALS AND METHODS

In vivo model.—The techniques for achieving reproducible 9L brain tumors in our laboratory has been reported (*8*). Briefly, male Fischer 344 rats, weighing 200–250 g, were anesthetized with sodium pentobarbital ip and placed (one at a time) in a small animal stereotactic apparatus. An incision was made in the skin over the right frontal bone, and a hole was bored with a 20-gauge needle through the skull exactly 4 mm from the sagittal suture and 1 mm posterior to the coronal suture. A 5-μl cell suspension (2.5×10^4 cells from monolayer culture) was injected at a depth of 3.5 mm beneath the skull with a 24-gauge needle directly into the white matter of the lobe. After the needle was removed, the hole was plugged with dental cement. Typically, this procedure resulted in 100% tumor take with untreated animals dying over approximately a 10-day range with MST between 20 and 28 days after inoculation.

A special apparatus was designed and built that allowed 5 animals to be restrained without anesthesia during whole-brain irradiation with a cesium-137 source. A dose rate of 7.3 Gy/min at the tumor site was determined by thermoluminescent dosimetry.

We obtained SPG as Spiro-32 (Unimed Co., Somerville, NJ) from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. The drug was diluted with sterile saline prior to use, and an appropriate dose in 1–2 ml saline was administered ip.

ABBREVIATIONS: SPG = spirogermanium; RT = radiation therapy; MST = median survival time(s); D_0 = dose increment required to reduce the surviving fraction by a factor of 1/e on the exponential portion of the dose-response curve; D_q = quasi-threshold dose.

---

[1] Radiation Biology Laboratory, Department of Radiation Oncology (*B. F. Kimler, D. F. Martin,* and *R. G. Evans*), and Section of Pediatric Oncology, Department of Pediatrics (*T. S. Vats*), University of Kansas Medical Center, Kansas City.

[2] Brain Tumor Institute of Kansas City, Research Medical Center, Kansas City, MO.

[3] We thank Gail G. Cox, David A. Kious, and Jorge Diaz for technical assistance.

* *Reprint requests:* Bruce F. Kimler, Ph.D., Radiation Biology Laboratory, Department of Radiation Oncology, University of Kansas Medical Center, Rainbow Blvd. at 39th St., Kansas City, KS 66103.

Data were analyzed with animal survival as the end point. We calculated the MST and tested for statistical significance using a Mann-Whitney test (*15*). When necessary, we also used a Gehan-Wilcoxon test for singly censored samples (*16*) to detect statistical differences in survival responses.

In vitro model.—The 9L/KC cells were maintained in exponential growth as monolayer cultures in McCoy's modified 5A medium supplemented with 10% fetal calf serum and (for experiments) antibiotics (100 U penicillin/ml; 100 μg streptomycin/ml). Cultures grown in this medium at 37° C in a humidified 5% CO_2 environment displayed a doubling time of 18 to 20 hours, with $G_1 = 9$–10 hours; $S = 5$–6 hours; $G_2 = 2$–3 hours; and $M = 1$ hour, as determined by labeling and mitotic indices (*17*).

The ability of cells to survive following drug treatment was assayed by the retention of colony-forming ability. Cells from exponentially growing populations were trypsinized and subsequently plated into 60-mm diameter culture dishes at cell concentrations such that between 50 and 200 colonies formed per plate after treatment. Five replicate plates were inoculated per data point. After cell inoculation, plates were incubated for at least 4 hours before treatment to allow cell attachment and recovery from trypsinization. After treatment, plates were returned to the incubator for 7–10 days. Colonies were stained with methylene blue, and the number of visible colonies of greater than 50 cells counted. Plating efficiency was calculated as the number of colonies formed divided by the number of cells inoculated; plating efficiencies of controls were typically in the range of 40%–60%. The surviving fraction after treatment was calculated as the plating efficiency following treatment divided by that of control plates; it was then plotted as a function of either SPG concentration or radiation dose. Error bars associated with the data points are standard deviations of the mean values from several repeat experiments. Determinations of extrapolation number and slope (and subsequent calculation of D_0 for the radiation dose–survival curves) were made from the line fit by a least-squares regression analysis (*15*).

For drug treatment, 1 ml of a concentrated drug solution was added to the medium already on the petri dishes so as to yield the appropriate drug concentration with minimal perturbation of the cells' growing conditions. For pulse exposures to the drug, the drug-containing medium was aspirated after the desired incubation time at 37° C and fresh medium was added.

Irradiations were performed at room temperature with the cesium-137 source at a dose rate of 4.5 Gy/minute. Typically, RT was administered in the middle of the drug exposure period.

RESULTS

In 2 experiments, SPG was injected ip 11 or 17 days after intracranial tumor inoculation at 50 or 60 mg/kg, doses estimated to be lethal to less than 10% of the rats. Of 59 animals that have been treated at these doses, 2 died of convulsive seizures within 2 hours of drug injection. These animals have been excluded from the analyses. In addition, numerous animals displayed symptoms of central nervous system toxicity, e.g., disorientation, seizures, convulsions, loss of bowel control, etc. Despite this evidence that the drug was reaching the CNS, SPG was ineffective in prolonging survival of tumor-burdened rats. As the responses to 50 and 60 mg/kg were virtually identical, the data from the 2 experiments have been combined (fig. 1). The MST of SPG-treated animals was 28 days, the same as for the controls. The 20 Gy of cesium-137 whole-head irradiation did prolong survival (MST = 36 days), which is statistically different from either controls or SPG ($P < .05$). However, when SPG was followed 1 hour later with RT, the survival response (MST = 33 days) was worse than that observed with RT alone and, in fact, was not statistically better than either control or SPG alone. The poorer survival seen with combined SPG and RT was not due to toxicity because no animal that survived more than 2 hours after a drug injection died before the first control animals died. Moreover, a dose of 20 Gy to rats without tumors is not toxic within 60 days. The maximum effective dose for prolonging MST is approximately 25 Gy; above that, MST decreases as toxicity becomes a factor. Combinations of single-dose SPG delivered 1 hour after RT or 1 day before RT were also tested. These were no more effective than RT alone in prolonging MST and in some cases were less effective than RT alone, but again they were not statistically so. These data indicate that SPG is ineffective against the 9L rat brain tumor when used in a single bolus injection and when added to single-dose RT does not improve survival compared with RT alone.

Because of the evidence that rats were being affected by neurotoxicity (numerous symptoms plus 2 rats dying immediately after treatment) when a bolus injection of SPG was given, a daily, fractionated treatment was attempted (fig. 2). Starting on day 15 after tumor inoculation, 5 daily doses of SPG (10 mg/kg per day) were delivered ip. This treatment resulted in an MST (22 days) that was not different from that seen in the control group (21 days). Another

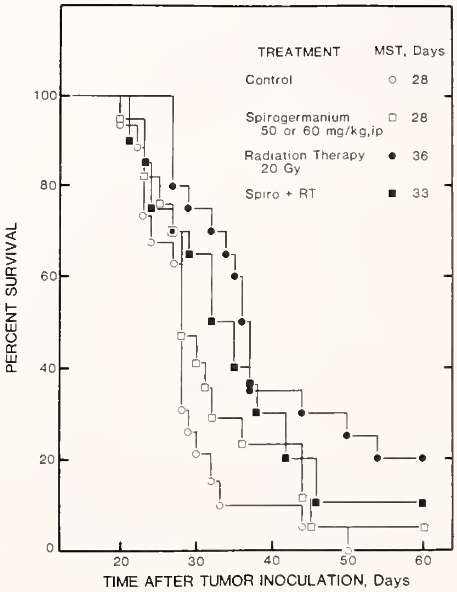


FIGURE 1.—Survival of 9L tumor-burdened rats treated as controls; with a single ip dose of 50 or 60 mg SPG/kg; with a single 20-Gy dose of whole-brain RT; or a single dose of SPG followed 1 hr later by a single dose of RT. Results from 2 experiments are combined. Treatment was initiated on day 11 or 17 after tumor inoculation.

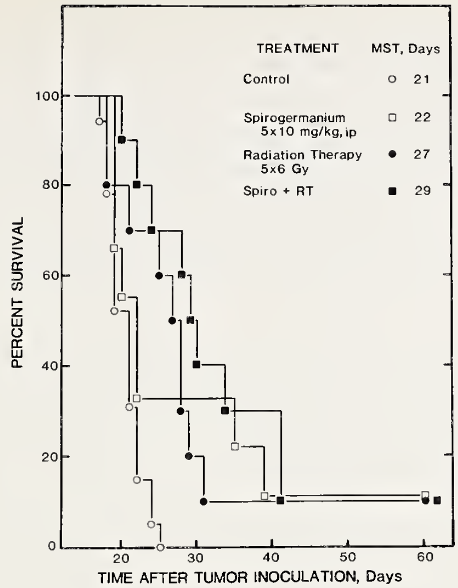


FIGURE 2.—Survival of 9L tumor-burdened rats treated as controls; with 5 daily ip doses of 10 mg SPG/kg; with 5 daily doses of 6 Gy of whole-brain RT; or the combination of 5 daily doses of SPG followed 1 hr later by 5 daily doses of RT. Treatment was initiated on day 15 after tumor inoculation.

group of animals was treated with 5 daily doses (6 Gy) of whole-brain RT which prolonged the MST (27 days). When each of the 5 daily doses of SPG (10 mg/kg) was followed 1 hour later by a daily dose of 6 Gy, there was a slight improvement in response (29 days) compared with that seen with RT only. However, this difference of 2 days was not statistically significant. Interestingly, although no significant ($P < .05$) differences were noted among any of the first 3 groups, the difference between the control and the combination treatment groups was significant ($P < .005$).

Finally, a schedule was tested that is similar to the clinical schedule currently in use against primary brain tumors. The maximum tolerable dose of 60 mg/kg was divided into 3 equal doses of 20 mg/kg and delivered on an every-other-day schedule starting on day 15 after tumor inoculation. With this schedule, SPG produced a statistically significant ($P < .05$) increase in MST (30 days) compared with the control MST of 24 days. We are currently testing whether this drug at this schedule can effectively enhance the effect of RT in prolonging MST.

The above results for single bolus injections of SPG are incompatible with the data that have been obtained for its effect on 9L cells in vitro, when colony formation was used as an index of cell survival. When used in 1-hour pulse exposures, SPG produced a concentration-dependent, exponential decrease in the surviving fraction (fig. 3). The mean lethal concentration (the inverse of the slope determined on the linear region of the survival curve) is 2.5 μg/ml (with a 95% confidence interval of 2.3–2.7 μg/ml). A concentration of 20 μg/ml for a 1-hour pulse exposure will reduce the surviving fraction below 0.1%, approximately the same level of survival as after 20 Gy. The in vivo concentration of SPG after a dose of 50 mg/kg (with conservative estimates for volume of distribution) should be two to four times this concentration. Even if the half-life of SPG in rat serum is only 15–20 minutes, there should still have been sufficient drug exposure to produce considerable cytotoxicity and hence prolongation of life span. Aside from the possibility that the drug does not penetrate the brain tumor itself, we have no explanation why SPG is so ineffective in this rat brain tumor model.

Figure 4 displays the radiation dose–survival curves of asynchronous 9L cells treated as controls or with SPG at various concentrations for 1-hour pulse exposures. The cytotoxicity as a result of drug alone was consistent with the data in figure 3. The radiation survival response of 9L cells is characterized by a wide shoulder ($D_q = 4.7$ Gy and $n = 9.8$) and a shallow slope, $D_0 = 2.1$ Gy (95% confidence interval of 1.7–2.7 Gy). Treatment with 2.5 μg SPG/ml produced essentially an additive effect on cell survival (product of cytotoxicity and radiation-induced cell lethality), which resulted in similar values for the survival curve parameters: $D_0 = 2.1$ Gy (1.7–2.7 Gy) and $D_q = 3.6$ Gy and $n = 5.7$ (corrected for drug-induced cytotoxicity). For the 5.0- and 7.5-μg SPG/ml treatments, there appeared to be a

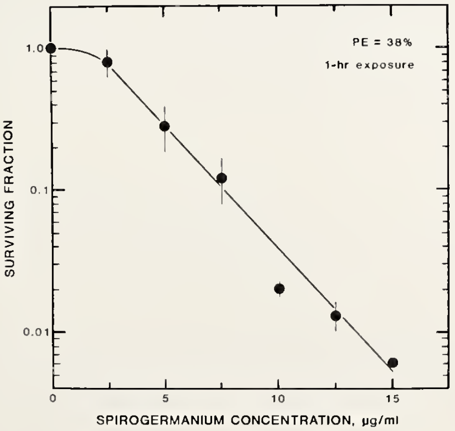


FIGURE 3.—Effect of 1-hr pulse exposures to SPG on the survival of 9L cells in monolayer culture as assayed by colony formation. A total of 6 experiments contribute to the data points. PE = plating efficiency.

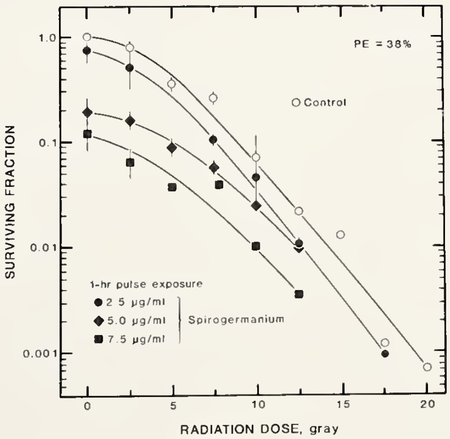


FIGURE 4.—Effect of 1-hr pulse exposures to SPG on the survival of irradiated 9L cells. A total of 5 experiments contribute to the data points.

less than additive effect on cell lethality at the higher radiation doses. However, the calculated values of D_0 (2.9 and 2.3 Gy, respectively) were not statistically significantly different than those obtained for the control and the cells treated with 2.5 μg SPG/ml.

DISCUSSION

It has been shown that SPG is effective at inducing cell lethality in asynchronous populations of 9L rat brain tumor cells in vitro. However, in the in vivo rat brain tumor model, SPG is ineffective at prolonging survival when used in single injections or daily schedules at doses that produce severe (occasionally fatal) neurotoxicity. So far, only one schedule (3 × 20 mg/kg, every other day) has produced an increased life span for tumor-burdened animals. Although in vitro there is an additive effect on induction of cell lethality by the combination of SPG and radiation, in vivo the combination treatment is not statistically better than RT alone. Whereas there is only a suggestion of enhancement with the daily schedule, it is possible that the every-other-day schedule might enhance RT because the SPG alone is effective. At this time, no explanation is available for the fact that SPG is effective against 9L cells in vitro and shows modest activity against human brain tumors in early clinical trials (*6*) but is ineffective in the 9L rat brain tumor model. Despite these questions, SPG when used in appropriate doses and schedules may still be a useful chemotherapy agent in combination with RT or other chemotherapy agents for the treatment and control of primary brain tumors. Therefore, additional studies in the 9L rat brain tumor model are being pursued.

REFERENCES

(*1*) Rice LM, Wheeler JW: Spirans. XXII. Synthesis of 4,4-dialkyl-4-germacyclohexanone and 8,8-dialkyl-8-germa-azaspiro(4,5)decanes. J Heterocycl Chem 11:1041–1047, 1974.

(*2*) Henry MC, Rosen E, Port CD, et al: Toxicity of spirogermanium in mice and dogs after iv or im administration. Cancer Treat Rep 64:1207–1210, 1980.

(*3*) Schwartz GN, Biegel JA, Fisher B, et al: Spirogermanium: Effects on hematopoietic stem cells and survival of normal and tumor-bearing mice. Proc Soc Exp Biol Med 173:176–180, 1983.

(*4*) Schein PS, Slavik M, Smythe T, et al: Phase I clinical trial of spirogermanium. Cancer Treat Rep 64:1051–1056, 1980.

(*5*) Slavik M, Blanc O, Davis J: Spirogermanium: A new investigational drug of novel structure and lack of bone marrow toxicity. Invest New Drugs 1:225–234, 1986.

(*6*) Goodwin JW, Crowley J, Tranum B, et al: A phase II trial of spirogermanium in central nervous system tumors. A Southwest Oncology Group study. Cancer Treat Rep 71:99–100, 1987.

(*7*) Wheeler KT, Barker MA, Wallen CA, et al: Evaluation of 9L as a brain tumor model. *In* Methods in Tumor Biology: Tissue Culture and Animal Tumor Models (Sridhar R, ed). New York: Marcel Dekker. In press.

(*8*) Henderson SD, Kimler BF, Morantz RA: Radiation therapy of 9L rat brain tumors. Int J Radiat Oncol Biol Phys 7:497–502, 1981.

(*9*) Barker M, Hoshino T, Gurcay O, et al: Development of an animal brain tumor model and its response to therapy with 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res 33:976–986, 1973.

(*10*) Vats TS, Morantz RA, Wood GW, et al: Study of effectiveness of bleomycin in rat brain tumor model intravenously and intracerebrally. Int J Radiat Oncol Biol Phys 5:1527–1529, 1979.

(*11*) Barker M, Deen DF, Baker DG: BCNU and X-ray therapy of intracerebral 9L rat tumors. Int J Radiat Oncol Biol Phys 5:1581–1583, 1979.

(*12*) Wheeler KT, Kaufman K: Influence of fractionation schedules on the response of a rat brain tumor to therapy with BCNU and radiation. Int J Radiat Oncol Biol Phys 6:845–849, 1980.

(*13*) Kimler BF, Vats TS, Morantz RA, et al: Response of the 9L rat brain tumor to combination treatment with radiation and bleomycin. Int J Radiat Oncol Biol Phys 7:1069–1074, 1981.

(*14*) Vats TS, Kimler BF, Henderson SD, et al: Combination chemotherapy for the 9L rat brain tumor model system. Cancer Treat Rep 66:575–579, 1982.

(*15*) Zar JH: Biostatistical Analysis. Englewood Cliffs, NJ: Prentice-Hall, 1974, pp 198–235.

(*16*) Gehan EA: A generalized Wilcoxon test for comparing arbitrarily singly censored samples. Biometrika 52:203–223, 1965.

(*17*) Kimler BF, Henderson SD: Cyclic response of cultured 9L cells to radiation. Radiat Res 91:155–168, 1982.

Potentiation of Rat Brain Tumor Therapy by Fluosol and Carbogen[1]

**Douglas F. Martin,[2,3,*] Bruce F. Kimler,[3] Richard G. Evans,[3] Robert A. Morantz,[2]
and Tribhawan S. Vats[3,4]**

ABSTRACT—We have been using the 9L rat brain tumor model to investigate the effect of the combination of a perfluorochemical emulsion, Fluosol-DA 20%, and carbogen breathing on the therapy of brain tumors. The combination of Fluosol, carbogen breathing, and carmustine (BCNU) was more effective at prolonging survival than was BCNU alone. This difference was small but significant ($P < .025$). Neither Fluosol without carbogen nor carbogen without Fluosol significantly altered the effect of BCNU. Fluosol and carbogen alone did not affect the survival of tumor-burdened rats. Fluosol and carbogen breathing did not alter the effect of single doses of radiation on these tumors. This result supports the hypothesis that 9L brain tumors contain few, if any, critical hypoxic cells. However, these tumors may contain cells which are oxygen deficient but not radiobiologically hypoxic. The Fluosol–carbogen combination may be changing the intratumor environment in such a way that the metabolism or activity of BCNU is altered.—NCI Monogr 6:119-122, 1988.

Despite recent advances in therapy, the prognosis for patients with high-grade, malignant brain tumors remains grim. Even with the best available therapy, the median survival time is still only about 1 year. Radiotherapy and nitrosoureas, particularly BCNU, produce some benefit but are limited by toxicity to the normal structures of the brain and the bone marrow, respectively.

Recently, researchers in several laboratories have begun extensive investigations of perfluorochemical emulsions as adjuncts to radiotherapy (*1–12*) and chemotherapy (*13–18*). Perfluorochemicals, hydrocarbons in which all hydrogen atoms have been replaced by fluorine, are generally able to dissolve unusually large amounts of oxygen (*19,20*). Physiologic emulsions of perfluorochemicals have been developed for intravascular use as oxygen transport fluids and plasma expanders (*20*). Experimental results indicate that administration of one such emulsion, Fluosol, when combined with oxygen or carbogen (95% O_2, 5% CO_2) breathing at ambient pressure, reduces the fraction of hypoxic cells in several experimental rodent tumors (*2,3,5–8,11*). This perfluorochemical is being widely examined in laboratory and clinical studies as an oxygen-transport agent and as an adjunct to both radiotherapy and chemotherapy. An extensive literature describing its use, toxicities, and radiobiologic effects is developing.

The rat 9L gliosarcoma has been used extensively in investigations of the effects of various therapeutic modalities on brain tumors, including BCNU (*21–24*), radiation therapy (*25*), and combinations of these modalities (*26–30*). We designed the experiments reported here to investigate whether the combination of Fluosol and carbogen breathing could potentiate the therapeutic effects of radiation therapy or BCNU on intracranial 9L tumors.

MATERIALS AND METHODS

The techniques for achieving reproducible 9L brain tumors in our laboratory have been reported (*25*). Briefly, male Fischer 344 rats, weighing 200–250 g, were anesthetized with sodium pentobarbital, and each rat was placed in a small animal stereotactic apparatus. An incision was made in the skin over the right frontal bone, and a hole was bored with a 23-gauge needle through the skull exactly 4 mm from the sagittal suture and 1 mm posterior to the coronal suture. A 5-μl cell suspension (2.5×10^4 cells from monolayer culture) was injected at a depth of 3.5 mm beneath the skull with a 24-gauge needle directly into the white matter of the lobe. The needle was removed and the hole was plugged with dental cement. Typically, this procedure results in 100% tumor take with untreated animals dying over approximately a 10-day range with MST between 18 and 28 days after tumor inoculation. Unless otherwise indicated, treatment was on day 15 after tumor inoculation. This time was chosen based on our considerations of tumor size, responsiveness to treatment, and time to death, if rats were left untreated. Although there may be a trend to a greater increase in life span when the tumor is treated at 9 versus 15 days, this difference is not significant. Ten rats were used in each treatment group.

Fluosol was manufactured by the Green Cross Corporation (Osaka, Japan) and was provided for these studies by Alpha Therapeutic Corporation (Los Angeles, CA). The composition and characteristics of the emulsion and the procedures for its formulation and administration have been described (*19,20*).

Fluosol was injected slowly into the tail veins of the rats at a dose of 10 ml/kg. This dose, which has been shown to be effective in enhancing the radiation response in other tumor systems (*2,7,9*), is comparable to the 8- to 9-ml/kg

---

ABBREVIATIONS: BCNU = carmustine; Fluosol = Fluosol-DA 20%; MST = median survival time(s).

---

[1] Supported in part by Alpha Therapeutic Corp., Los Angeles, CA.

[2] The Brain Tumor Institute of Kansas City, Research Medical Center, MO.

[3] Radiation Biology Laboratory, Department of Radiation Oncology (*D. F. Martin, B. F. Kimler,* and *R. G. Evans*), and Section of Pediatric Oncology, Department of Pediatrics (*T. S. Vats*), University of Kansas Medical Center, Kansas City.

[4] We thank Gail G. Cox, David A. Kious, and Jorge Diaz for technical assistance.

[*] *Reprint requests:* Douglas F. Martin, Ph.D., Radiation Biology Laboratory, Department of Radiation Oncology, University of Kansas Medical Center, Rainbow Blvd. at 39th St., Kansas City, KS 66103.

dose (*12*) currently being used in clinical protocols. The BCNU was obtained from Bristol Laboratories (Syracuse, NY) and was injected ip as a single dose of 10 mg/kg.

A special apparatus was designed and built that allowed 5 animals to be restrained without anesthesia during whole-head or whole-brain irradiation with a cesium-137 source at 7.3 Gy/minute. While the rats were in the irradiation chamber, the appropriate gas, either air or carbogen, was passed through the chamber at 15 liters/minute. In the BCNU experiments, rats treated with carbogen breathing were held in a 45-×45-×60-cm chamber with carbogen flowing through at 15 liters/minute.

Data were analyzed with animal survival as the end point. We calculated the MST and tested for statistical significance using a Mann-Whitney test, or when necessary, a Gehan-Wilcoxon test for singly censored samples (*31*).

RESULTS

We tested the ability of Fluosol and carbogen breathing to enhance the response of intracranial 9L tumors to therapy with BCNU using MST as an assay of therapeutic effectiveness. Tumor-burdened rats were given injections of 10 ml Fluosol/kg, placed in carbogen for 15 minutes, given an injection of BCNU, and returned to carbogen for 3 hours. In every experiment, every group treated with BCNU, either alone or in combination with Fluosol and/or carbogen breathing, had a significantly ($P<.05$) greater survival than did the control group. This is consistent with previous findings that BCNU is effective against 9L brain tumors (*21–24*).

In 5 of 6 experiments, the survival of rats treated with the combination of Fluosol, BCNU, and carbogen breathing was prolonged relative to rats treated with BCNU alone. However, these differences were small. In 1 experiment, the difference was significant ($P<.05$) by the Gehan–Wilcoxon test. In 3 others, the difference was borderline ($.10>P>.05$). When the data were combined (fig. 1), the MST for rats treated with the combination was 42 days and was significantly different ($P<.025$) from the MST of 35 days for rats treated with BCNU alone.

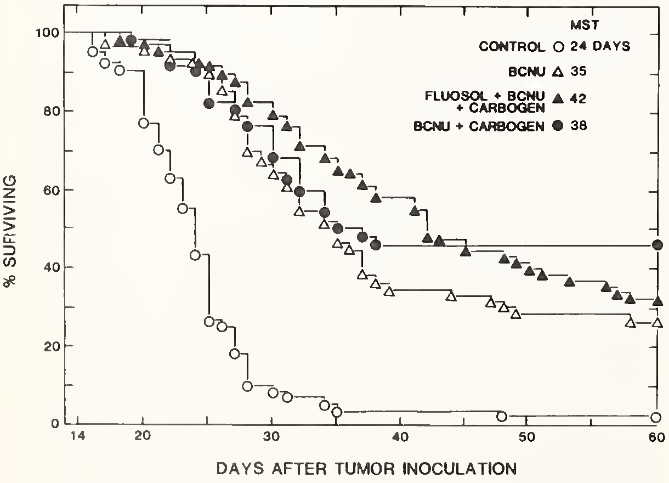


FIGURE 1.—Survival of rats with intracranial 9L tumors. Rats were treated with 10 mg BCNU/kg ip; BCNU and carbogen breathing (15 min before BCNU and 3 hr after); 10 ml Fluosol/kg iv followed immediately by 15 min of carbogen breathing, BCNU, and 3 hr carbogen breathing; or left untreated as controls. Results of 4 (BCNU and carbogen breathing) or 6 (all other treatments) experiments are combined.

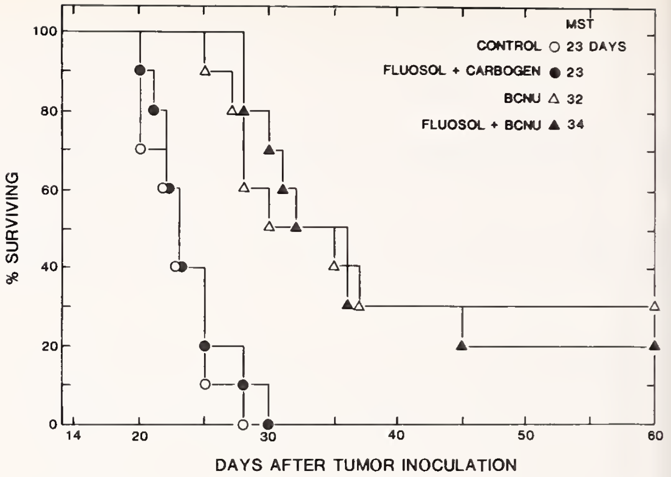


FIGURE 2.—Survival of rats with intracranial 9L tumors. Rats were treated with 10 mg BCNU/kg ip; with 10 ml Fluosol/kg iv, and BCNU 15 min later; with Fluosol followed immediately by carbogen breathing for 3 hr and 15 min; or left untreated as controls. Treatment was on day 15 after tumor inoculation.

Carbogen breathing without Fluosol did not produce the same enhancement of BCNU therapy as did the Fluosol–carbogen combination (fig. 1). The MST for rats treated with BCNU and carbogen breathing (36 days) was essentially the same as the MST for rats treated with BCNU alone (35 days). Although it was smaller than the MST for rats treated with the BCNU–Fluosol–carbogen combination (42 days), the difference was not significant. The combination of Fluosol and carbogen without BCNU did not alter the MST of tumor-burdened rats (MST were the same as for controls, i.e., 23 days; fig. 2). Likewise, Fluosol without carbogen did not enhance the effect of BCNU on 9L tumors. The MST were 34 days for rats treated with Fluosol and BCNU compared with 32 days for rats treated with BCNU alone (fig. 2). These results have been repeated.

Overall, our findings support the hypothesis that the combination of Fluosol and carbogen breathing increases the oxygenation of 9L intracranial tumors in such a manner that the effect of BCNU therapy is enhanced.

The combination of Fluosol and carbogen breathing was also tested for its ability to enhance the outcome of radiation therapy of 9L tumors. We gave the tumor-burdened rats injections of 10 ml Fluosol/kg and then placed them in carbogen for 15 minutes before and during irradiation. In several experiments, at doses ranging from 12 to 25 Gy, no enhancement of the radiation response was observed (fig. 3).

DISCUSSION

Fluosol enhances the radiation response of a variety of experimental tumors when combined with carbogen breathing at 1 atmosphere (*1–8*) or with hyperbaric oxygen (*9*). This effect is thought to be due to increased oxygenation and a concomitant decrease in the hypoxic fraction of the tumor. The Fluosol–carbogen combination has also been

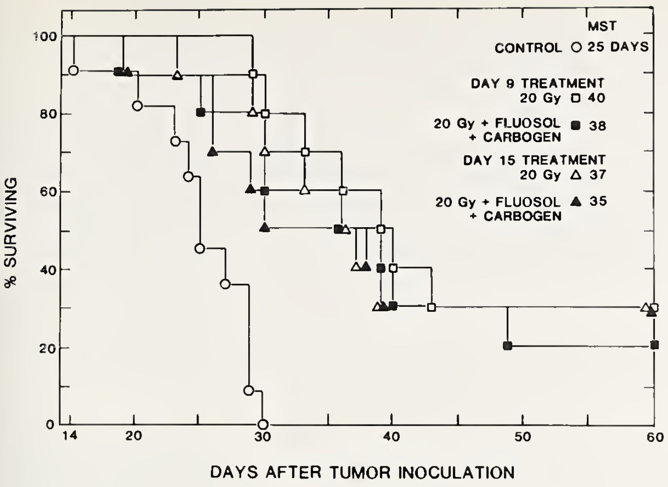


FIGURE 3.—Survival of rats with intracranial 9L tumors. Rats were treated with 20 Gy radiation; Fluosol followed by 15 min of carbogen breathing and 20 Gy radiation (carbogen breathing was continued during irradiation); or left untreated as controls. Treatment was on day 9 or 15 after tumor inoculation.

found to enhance the antitumor effects of some chemotherapeutic drugs including several nitrosoureas (*13–18*). The data presented in this report demonstrate that in the 9L rat brain tumor model Fluosol and carbogen breathing result in a small but significant potentiation of the therapeutic effect of BCNU but not of radiation.

The question of whether there are hypoxic cells in 9L tumors remains unresolved. The previously published radiation survival curve data for 9L cells treated in vivo are ambiguous and can be interpreted to show a small number of fully hypoxic cells, many cells at an intermediate level of hypoxia, or no hypoxic cells (*32*). The electron-affinic compound misonidazole is an effective radiosensitizer of hypoxic 9L cells in culture (*33*), but the addition of misonidazole to radiation therapy protocols, either single dose or fractionated, does not improve the MST of 9L tumor-burdened rats (*34*). The low level of binding of misonidazole, a measure of hypoxia, found in subcutaneous 9L tumors is inconsistent with the presence of fully hypoxic cells (*35*). Furthermore, 9L cells in culture consume oxygen much more slowly than do other rodent tumor cells (*35*). Thus the bulk of the evidence suggests that 9L tumors have few if any critical hypoxic cells. The finding that the combination of Fluosol and carbogen does not enhance the radiation response of intracranial 9L tumors is consistent with a lack of critical hypoxic cells.

Several possible mechanisms other than the presence of hypoxic cells may explain the potentiation of BCNU therapy of 9L tumors. Teicher et al. (*18*) have commented that nitrosoureas are cytostatic agents and suggested that treatment with Fluosol, carbogen breathing, and a nitrosourea can cause a larger proportion of cells to enter a quiescent state than would treatment with the nitrosourea alone. Using the FSa-IIc fibrosarcoma in mice, they found that the Fluosol–carbogen combination enhances the antitumor effect of nitrosoureas as assayed by tumor cell kill and growth delay. However, the cell killing was not as great as expected from the growth delay results. This finding is consistent with the hypothesis that the cytostatic effect of the nitrosoureas is enhanced by Fluosol and carbogen breathing.

Other possible mechanisms which might account for the small enhancement of BCNU activity by Fluosol and carbogen include activation of an immune component (*11*) or changes in drug delivery (pharmacokinetics). The latter possibility could not explain the importance of carbogen breathing. In fact, the lack of any effect of Fluosol without carbogen breathing argues against a drug delivery mechanism.

In the absence of critical radiobiologically hypoxic cells, increased oxygen delivery by Fluosol could affect BCNU therapy if a significant fraction of cells were in a low oxygen environment. The increased oxygen delivery could stimulate these cells into an enhanced metabolic state, analogous to feeding a starved plateau-phase cell culture. The stimulated cells would be expected to have an increased sensitivity to BCNU. Alternatively, the increased oxygen delivery could induce changes in the intracellular environment (e.g., increase the pH) such that the cytotoxicity of the drug would be increased.

Treatment with Fluosol results in manageable toxicities (*5,10,15,18,20*). The Fluosol–carbogen treatment produces only small changes in the survival curves of intestinal epithelial cells and spermatogenic stem cells of mice irradiated in vivo (*10*). The combination of Fluosol and carbogen at 1 atmosphere does not alter the radiation response of the hematopoietic stem cells of mouse bone marrow or spleen and produces only a slight increase in skin reactions in irradiated mouse feet (*5*). In addition, the Fluosol–carbogen combination does not increase the killing of mouse bone marrow cells by nitrosoureas (*18*), nor does it alter the production of artificial metastases by EMT6 cells (*4*). The finding of only minimal toxicities combined with the slight radiosensitization and chemosensitization of normal tissues produced by treatment with Fluosol and carbogen breathing suggests that this combination produces a therapeutic gain when used as an adjunct to cancer therapy.

REFERENCES

(*1*) FISCHER JJ, ROCKWELL S, MARTIN DF: Perfluorochemicals and hyperbaric oxygen in radiation therapy. Int J Radiat Oncol Biol Phys 12:95–102, 1986.

(*2*) ROCKWELL S: Use of a perfluorochemical emulsion to improve oxygenation in a solid tumor. Int J Radiat Oncol Biol Phys 11:97–103, 1985.

(*3*) ROCKWELL S, FISCHER JJ, MARTIN DF: Use of perfluorochemical emulsions to improve oxygenation in solid tumors. *In* Proceedings of the Seventh International Congress of Radiation Research, Amsterdam, July 3–9, 1983.

(*4*) ROCKWELL S, IRVIN CG, NIERENBURG M: Effect of a perfluorochemical emulsion on the development of artificial lung metastases in mice. Exp Clin Metastasis 4:45–50, 1986.

(*5*) ROCKWELL S, MATE TP, IRVIN CG, et al: Reactions of tumors and normal tissues in mice to irradiation in the presence and absence of a perfluorochemical emulsion. Int J Radiat Oncol Biol Phys 12:1315–1318, 1986.

(*6*) SONG CW, ZHANG WL, PENCE DM, et al: Increased radiosensitivity of tumors by perfluorochemicals and carbogen. Int J Radiat Oncol Biol Phys 11:1833–1836, 1985.

(*7*) TEICHER BA, ROSE CM: Oxygen-carrying perfluorochemical emulsion as an adjuvant to radiation therapy in mice. Cancer Res 44:4285–4288, 1984.

(*8*) Teicher BA, Rose CM: Perfluorochemical emulsions can increase tumor radiosensitivity. Science 223:934–936, 1984.

(*9*) Martin DF, Porter EA, Rockwell S, et al: Enhancement of tumor radiation response by the combination of a perfluorochemical emulsion and hyperbaric oxygen. Int J Radiat Oncol Biol Phys 13:747–751, 1987.

(*10*) Mason KA, Withers HR, Steckel RJ: Acute effects of a perfluorochemical oxygen carrier on the normal tissues of the mouse. Radiat Res 104:387–394, 1985.

(*11*) Teicher BA, McIntosh NL, Rose CM: Evidence for an immune component in the enhancement of tumor growth delay with X-ray/Fluosol-DA/O_2 combination therapy. Radiat Res. In press.

(*12*) Rose C, Lustig R, McIntosh N, et al: A clinical trial of Fluosol-DA 20% in advanced squamous cell carcinoma of the head and neck. Int J Radiat Oncol Biol Phys 12:1325–1327, 1986.

(*13*) Kokunai T, Kuwamura K: Effect of perfluorochemicals on BCNU chemotherapy: Preliminary study in a rat brain tumor model. Surg Neurol 18:258–261, 1982.

(*14*) Kuwamura T, Kokunai T, Tamaki N, et al: Synergistic effect of perfluorochemicals on BCNU chemotherapy. Experimental study in a 9L rat brain-tumor model. J Neurosurg 57:467–471, 1982.

(*15*) Teicher BA, Holden SA, Rose CM: Differential enhancement of melphalan cytotoxicity in tumor and normal tissue by Fluosol-DA and oxygen breathing. Int J Cancer 36:585–589, 1985.

(*16*) Teicher BA, Holden SA, Rose CM: Effect of oxygen on the cytotoxicity and antitumor activity of etoposide. JNCI 75:1129–1133, 1985.

(*17*) Teicher BA, Rose CM: Enhancement of anticancer drug efficacy by a perfluorochemical emulsion (PFC). Proc Am Assoc Cancer Res 25:316, 1984.

(*18*) Teicher BA, Holden SA, Rose CM: Effect of Fluosol-DA/O_2 on tumor-cell and bone-marrow cytotoxicity of nitrosoureas in mice bearing FSa-II fibrosarcoma. Int J Cancer 38:285–288, 1986.

(*19*) Clark LC, Gollan F: Survival of mammals breathing organic liquids equilibrated with oxygen at atmospheric pressure. Science 152:1755–1756, 1966.

(*20*) Suyama T, Yokoyama K, Naito R: Development of a perfluorochemical whole blood substitute (Fluosol-DA, 20%). An overview of clinical studies with 185 patients. *In* The Red Cell (Brewer GJ, ed). New York: Alan R Liss, 1981, pp 609–626.

(*21*) Barker M, Hoshino T, Gurcay O, et al: Development of an animal brain tumor model and its response to therapy with 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res 33:976–986, 1973.

(*22*) Rosenblum ML, Wheeler KT, Wilson CB, et al: In vitro evaluation of in vivo brain tumor chemotherapy with 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res 35:1387–1391, 1975.

(*23*) Tel E, Barker M, Levin VA, et al: Effect of combined surgery and BCNU (NSC-409962) on an animal brain tumor model. Cancer Chemother Rep 58:627–631, 1974.

(*24*) Levin VA, Kabra P: Effectiveness of the nitrosoureas as a function of their lipid solubility in the chemotherapy of experimental rat brain tumors. Cancer Chemother Rep 58:787–792, 1974.

(*25*) Henderson SD, Kimler BF, Morantz RA: Radiation therapy of 9L rat brain tumors. Int J Radiat Oncol Biol Phys 7:497–502, 1981.

(*26*) Barker M, Deen DF, Baker DG: BCNU and X-ray therapy of intracerebral 9L rat tumors. Int J Radiat Oncol Biol Phys 5:1581–1583, 1979.

(*27*) Rosenblum ML, Dougherty DA, Wilson CB: Rational planning of brain tumour therapy based on laboratory investigations: Comparison of single- and multiple-dose BCNU schedules. Br J Cancer 41(suppl IV):253–254, 1980.

(*28*) Wheeler KT, Barker MA, Wallen CA, et al: Evaluation of 9L as a brain tumor model. *In* Methods in Tumor Biology: Tissue Culture and Animal Tumor Models (Sridhar R, ed). New York: Marcel Dekker. In press.

(*29*) Wheeler KT, Kaufman K: Influence of fractionation schedules on the response of a rat brain tumor to therapy with BCNU and radiation. Int J Radiat Oncol Biol Phys 6:845–849, 1980.

(*30*) Wheeler KT, Kaufman K, Feldstein M: Response of a rat brain tumor to fractionated therapy with low doses of BCNU and irradiation. Int J Radiat Oncol Biol Phys 5:1553–1557, 1979.

(*31*) Gehan EA: A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. Biometrika 52:203–223, 1965.

(*32*) Moulder JE, Rockwell S: Hypoxic fractions of solid tumors: Experimental techniques, methods of analysis, and a survey of existing data. Int J Radiat Oncol Biol Phys 10:695–712, 1984.

(*33*) Henderson SD, Kimler BF: Hypoxic radiosensitization of 9L cells in vitro by misonidazole. *In* Radiation Sensitizers: Their Use in the Clinical Management of Cancer (Brady LW, ed). New York: Masson, 1980, pp 455–457.

(*34*) Reddy EK, Kimler BF, Henderson SD, et al: Combination misonidazole and radiation therapy in a rat brain tumor (9L) system. *In* Radiation Sensitizers: Their Use in the Clinical Management of Cancer (Brady LW, ed). New York: Masson, 1980, pp 457–460.

(*35*) Franco AJ, Koch CJ, Garrecht BM, et al: Hypoxia in 9L spheroids and tumors: Misonidazole binding and oxygen consumption. Presented at the 34th Annual Meeting of the Radiation Research Society, Las Vegas, NV, April 12–17, 1986.

Plasma and Tumor Concentrations of Cisplatin Following Intraperitoneal Infusion or Bolus Injection With or Without Continuous Low-dose-rate Irradiation[1,2]

Karen K. Fu,* Michael W. DeGregorio, and John W. Phillips[3]

ABSTRACT—Our purpose of this study was to determine whether whole-body, continuous low-dose-rate irradiation (CLDRI) alters the plasma and/or tumor platinum pharmacokinetics after ip bolus injection or ip infusion as a possible mechanism of interaction between CLDRI and cisplatin. The C3Hf/Sed mice bearing SCCVII/SF tumors were given 6 mg cisplatin/kg ip by bolus injection or an ip infusion of 0.25 mg cisplatin·kg^{-1}·hour^{-1} for 48 hours with and without CLDRI at 0.56 Gy/hr for 24 or 48 hours. Plasma and tumor platinum concentrations were determined with an atomic absorption spectrophotometer at appropriate intervals during infusion and up to 48 hours after drug administration. Both total and ultrafilterable plasma platinum followed a biphasic elimination after ip bolus injection, whereas only a prolonged single-phase elimination was seen after ip infusion. Tumor uptake of platinum appeared to follow a passive diffusion pattern with a prolonged cellular retention of platinum. Whole-body CLDRI had no apparent effect on the pharmacokinetics of plasma and tumor platinum administered by ip bolus injection or prolonged continuous infusion.—NCI Monogr 6:123–127, 1988.

Cisplatin has been combined with radiation in the treatment of head and neck, bladder, brain, esophageal, cervical, lung, and ovarian cancers (*1–6*). In experimental studies, cisplatin has been shown to enhance the effects of radiation on tumors as well as normal tissues (*7–14*).

We (*12*) have shown that cisplatin alone was more effective against the SCCVII/SF tumor when given by ip bolus injection than by prolonged continuous infusion. However, when cisplatin was combined with CLDRI, the combined effects were additive with cisplatin administered by bolus injection after CLDRI and supra-additive with concurrent cisplatin infusion and CLDRI. We conducted the present study to determine the effect of CLDRI on plasma and tumor cisplatin pharmacokinetics following different schedules of administration as part of our investigation of the possible mechanisms of interaction between CLDRI and cisplatin.

MATERIALS AND METHODS

Mice and tumor.—Ten-week-old C3Hf/Sed male mice (obtained from the Edwin L. Steele Laboratory, Department of Radiation Medicine, Harvard Medical School, Boston, MA) with an average weight of 30 g were used in all experiments.

The SCCVII/SF tumor, a spontaneous squamous cell carcinoma, was transplanted subcutaneously in the flanks of the mice. The tumors were treated in vivo when they reached a mean diameter of 8 ± 1 mm.

Drug administration.—Cisplatin (Bristol Laboratories, Syracuse, NY) was dissolved and diluted in sterile water immediately before use. For continuous infusion, cisplatin was infused through an intraperitoneally implanted Alzet osmotic minipump (model 2001, from Alza Corporation, Palo Alto, CA) at a rate of 0.25 mg·kg^{-1}·ml^{-1}·hr^{-1}. With the mice under brief general anesthesia induced by methoxyflurane, the osmotic minipumps were implanted in the peritoneal cavity 1 hour prior to the beginning of CLDRI and removed immediately after the end of whole-body CLDRI and/or chemotherapy infusion (total cisplatin dose was 12.25 mg/kg in 49 hr).

For bolus injection, cisplatin at a dose of 6 mg/kg was injected ip in a volume of 0.01 ml/g body weight. In the combined treatment group, the drug was injected ip immediately after whole-body CLDRI at 0.56 Gy/hour for 24 hours.

Irradiation procedures.—The method of CLDRI has been described (*15*). Tumor-bearing mice were exposed to whole-body CLDRI at 0.56 Gy/hour for 24 or 48 hours in a cesium-137 laboratory irradiator (Mark I model 68, cesium-137 irradiator; J. L. Sheperd & Assoc., Glendale, CA).

Pharmacokinetic studies.—Total and ultrafilterable plasma platinum and total tumor platinum concentrations were determined with a flameless atomic absorption spectrophotometer assay as described (*16*). Briefly, a spectrophotometer (Perkin Elmer model 2280; The Perkin Elmer Corp., Norwalk, CT) with an HGA 400 graphite furnace was standardized with a stock solution of cisplatin (correlation coefficient >0.99); the spectrophotometer was set to measure absorbance at 265.9 nm with deuterium background correction.

ABBREVIATION: CLDRI = continuous low-dose-rate irradiation.

[1] Supported by grant No. PDT-289 from the American Cancer Society and by the Louis R. Lurie and Randy Lynn Baruh Foundations (*M. W. DeGregorio*).

[2] Cisplatin was supplied by the Bristol-Myers Co.

[3] Department of Radiation Oncology (*K. K. Fu* and *J. W. Phillips*) and Cancer Pharmacokinetics Laboratory, Children's Cancer Research Institute, Pacific Presbyterian Medical Center and Cancer Research Institute, (*M. W. DeGregorio*), University of California, San Francisco.

* *Reprint requests:* Karen K. Fu, M.D., Department of Radiation Oncology, Rm. L-75, University of California, San Francisco, San Francisco, CA 94143.

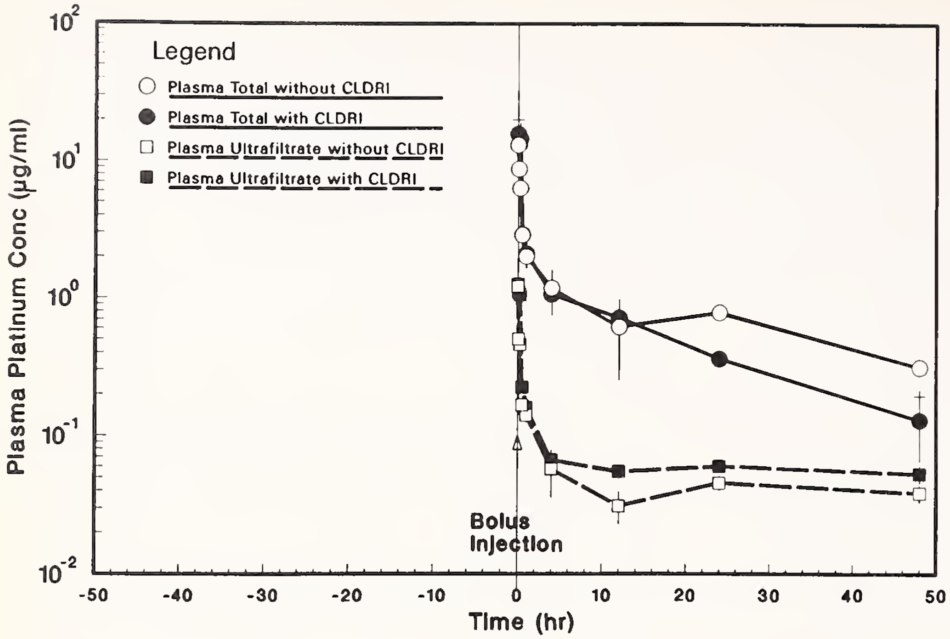


FIGURE 1.—Plasma platinum concentration with bolus injection at 6 mg/kg ip. All values are averages of 6 determinations. *Symbols* represent the mean of 2 experiments; *bars* represent SEM.

Plasma and tumor samples were collected at 7, 17, 25, and 49 hours after the beginning of continuous infusion, at 5, 15, and 30 minutes, and 1, 4, 12, 24, and 48 hours after the end of infusion or following bolus injection. Whole blood was obtained by jugular phlebotomy with the animal under general anesthesia. Plasma was separated from whole blood by centrifugation at 600 *g* for 10 minutes. We prepared the plasma ultrafiltrate by using Amicon CF-30 filters and centrifugation at 5,000 *g* for 20 minutes. Following whole blood sample collections, the animals were euthanized and the tumors were excised and weighed in preweighed glass tubes. Each tumor sample was completely dissolved in a known volume of nitric acid. Total volumes of the dissolved samples were then measured and 20-µl injections were analyzed for platinum concentrations.

RESULTS

Total and ultrafilterable plasma platinum for bolus injection and continuous ip infusion are shown in figures 1 and 2. Peak plasma platinum concentrations were achieved within 25 hours after the start of continuous infusion or immediately after bolus injection. Both total and ultrafilterable plasma platinum followed a biphasic elimination after bolus administration, whereas only a prolonged single-phase elimination was seen after ip infusion. Table 1 shows the half-lives of ultrafilterable plasma platinum. Whole-body CLDRI had no significant effect on the pharmacokinetics of plasma platinum administered by ip infusion or bolus injection.

Tumor platinum concentrations for ip bolus injection

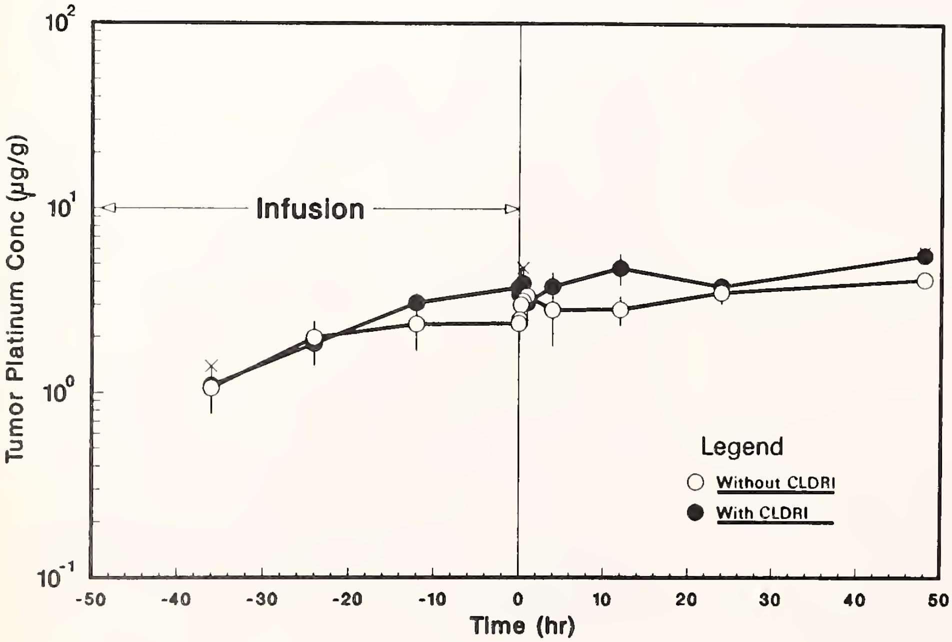


FIGURE 2.—Plasma platinum concentration with ip infusion at 0.25 mg·kg^{-1}·hr^{-1} for 49 hr. All values are averages of 6 determinations. *Symbols* represent the mean of 2 experiments; *bars* represent SEM.

TABLE 1.—Half-lives of plasma ultrafilterable platinum following ip bolus injection or continuous infusion with or without CLDRI[a]

Half-lives	With CLDRI, hr	Without CLDRI, hr
Bolus injection		
Initial	0.34	0.31
Terminal	29.2	27.7
Infusion, ip		
Terminal	29.3	30.0

[a] All values were averages of duplicate experiments and were within 10% variation.

and continuous infusion are shown in figures 3 and 4. Peak platinum levels were achieved immediately after the end of drug administration. The uptake of platinum appeared to follow a passive diffusion pattern which can be predicted when peak tumor platinum concentrations correspond to peak plasma levels. The prolonged cellular retention of platinum, as shown by the minimal changes in tumor platinum concentrations, is consistent with other reports that demonstrate that cisplatin complexes have long tissue half-lives (*17–19*). However, accurate determination of the prolonged half-lives of elimination for tumor platinum was not possible with the available data because our measurements were done only up to 48 hours after drug administration. Similar to plasma platinum concentrations, whole-body CLDRI had no apparent effect on the tumor uptake of platinum.

The area under the concentration versus time curves for plasma and tumor with and without CLDRI are shown in table 2. Although minor differences exist, no consistent pattern showing a pharmacokinetic advantage was seen between irradiated and nonirradiated mice.

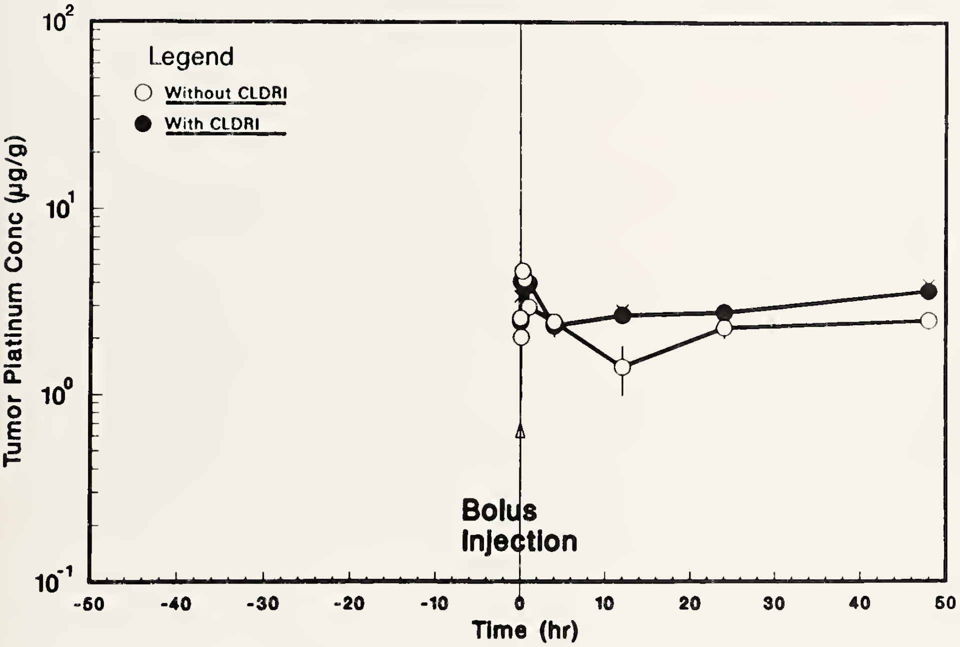


FIGURE 3.—The SCCVII/SF tumor platinum concentration with bolus injection at 6 mg/kg ip. *Symbols* represent the mean of 4 tumors; *bars* represent SEM.

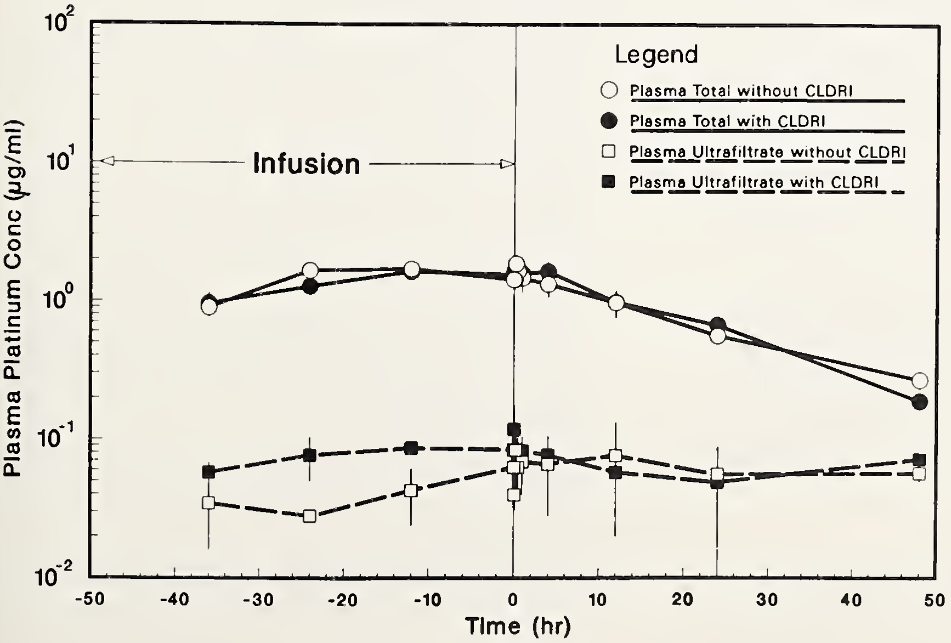


FIGURE 4.—The SCCVII/SF tumor platinum concentration with ip infusion at 0.25 mg·kg^{-1}·hr^{-1} for 49 hr. *Symbols* represent the mean of 4 tumors; *bars* represent SEM.

TABLE 2.—Area under the curve for plasma and tumor platinum 0–48 hr after ip bolus injection or continuous infusion with and without CLDRI

	Area under the curve[a]		
	Plasma platinum $\mu g \cdot hr^{-1} \cdot ml^{-1}$		Tumor platinum, $\mu g \cdot hr^{-1} \cdot g^{-1}$
Treatment[b]	Total	Ultrafiltrate	Total
Bolus injection			
With CLDRI	36.3	3.4	142.5
Without CLDRI	39.7	2.2	107.1
Infusion, ip			
With CLDRI	27.0	1.5	99.5
Without CLDRI	34.5	3.2	164.8

[a] All values were averages of duplicate experiments and were within 15% variation.

[b] Bolus injection was 6 mg cisplatin/kg; ip infusion was 12.25 mg cisplatin/kg.

DISCUSSION

Cisplatin has been shown to enhance the radiation effects in tumor and normal tissues in animal studies (*7–14*). The mechanism of interaction is not fully understood. Possible mechanisms of interaction include: 1) radiosensitization of hypoxic cells (*20–22*), 2) inhibition of repair of sublethal and potentially lethal damage (*8,12,14,21,23*), 3) induction of DNA cross-links (*24,25*), 4) depletion of intracellular thiols (*7*), and 5) altered drug pharmacokinetics.

Several studies of the plasma kinetics of different cisplatin infusion lengths in humans and animals have shown that: 1) Plasma platinum is highly protein bound; 2) total plasma platinum is eliminated in a biphasic kinetic pattern; 3) ultrafilterable platinum represents the active platinum complexes, and these complexes are eliminated either by a single or biphasic kinetic pattern (*26–31*); and 4) the active platinum complexes are eliminated within a short period after cisplatin therapy. In our study, total and ultrafilterable plasma platinum display curves and kinetic parameters for ip bolus injection and 49-hour infusions with and without CLDRI were consistent with these reports.

Tissue distribution of platinum after cisplatin infusion has been studied by several investigators (*17–19*). The uptake by most tissues appears to be rapid and is then followed by a long cellular retention of the platinum complexes. In agreement with these studies, we demonstrated similar kinetic patterns in which platinum complexes were rapidly taken up by tumor cells followed by a long cellular retention time.

In summary, we detected no significant difference in plasma or tumor pharmacokinetics for total and ultrafilterable platinum complexes with ip bolus injection and continuous infusion, with or without whole-body CLDRI. Thus the previously observed supra-additive effect when cisplatin was given by continuous infusion during the course of CLDRI cannot be explained on the basis of altered cisplatin pharmacokinetics.

REFERENCES

(*1*) AL-SARRAF M, KINZIE J, MARCIAL V, et al: High local and regional complete remission with the combination of *cis*-platinum and radiotherapy in unresectable head and neck cancers. Proc ASCO 4:50, 1985.

(*2*) SHIPLEY W, COOMBS J, EINSTEIN A, et al: Cisplatin and full dose irradiation for patients with invasive bladder carcinoma: A preliminary report of tolerance and local response. J Urol 132:899–903, 1984.

(*3*) LEICHMAN L, STEIGER Z, SEYDEL HG, et al: Preoperative chemotherapy and radiation therapy for patients with cancer of the esophagus: A potentially curative approach. J Clin Oncol 2:75–79, 1984.

(*4*) TWIGGS LB, POTISH RA, MCINTYRE S, et al: Concurrent weekly *cis*-platinum and radiotherapy in advanced cervical cancer: A preliminary dose escalating toxicity study. Gynecol Oncol 24:143–148, 1986.

(*5*) COLTART RS, NETHERSELL AB, BROWN CH: A pilot study of high dose abdominopelvic radiotherapy following surgery and chemotherapy for stage III epithelial carcinoma of the ovary. Gynecol Oncol 23:105–110, 1986.

(*6*) SCHAAKE KC, BARTELINK H, ADEMA BH, et al: Radiotherapy and *cis*-diammine dichloroplatinum (II) as a combined treatment modality for inoperable non-small cell lung cancer: A dose finding study. Int J Radiat Oncol Biol Phys 12:379–383, 1986.

(*7*) BARTELINK H, KALLMAN RF, RAPACCHIETTA D, et al: Therapeutic enhancement in mice by clinically relevant dose and fractionation schedules of *cis*-diamminedichloroplatinum (II) and irradiation. Radiother Oncol 6:61–74, 1986.

(*8*) BURHOLT DR, SCHENKEN LL, KOVACS CJ, et al: Response of the murine gastrointestinal epithelium to *cis*-dichlorodiammineplatinum II: Radiation combinations. Int J Radiat Oncol Biol Phys 5:1377–1381, 1979.

(*9*) DOUPLE EB, EATON WL, TULLOH ME: Skin radiosensitization studies using combined *cis*-dichlorodiammineplatinum II and radiation. Int J Radiat Oncol Biol Phys 5:1383–1385, 1979.

(*10*) DOUPLE EB, RICHMOND RC: Enhancement of the potentiation of radiotherapy by platinum drugs in a mouse tumor. Int J Radiat Oncol Biol Phys 8:501–503, 1982.

(*11*) OVERGAARD J, KHAN AR: Selective enhancement of radiation response in C3H mammary carcinoma with cisplatin. Cancer Treat Rep 65:501–503, 1981.

(*12*) FU KK, LAM KN, RAYNER PA: The influence of time sequence of cisplatin administration and continuous low dose rate irradiation (CLDRI) on their combined effects on a murine squamous cell carcinoma. Int J Radiat Oncol Biol Phys 11:2119–2124, 1985.

(*13*) LELIEVELD P, SCOLES MA, BROWN JM, et al: The effect of treatment in fractionated schedules with the combination of X-irradiation and six cytotoxic drugs on the RIF-1 tumor and normal mouse skin. Int J Radiat Oncol Biol Phys 11:111–121, 1985.

(*14*) LUK KH, ROSS GY, PHILLIPS TL, et al: The interaction of radiation and *cis*-diamminedichloroplatinum (II) in intestinal crypt cells. Int J Radiat Oncol Biol Phys 5:1417–1420, 1979.

(*15*) FU KK, PHILLIPS TL, KANE LJ, et al: Tumor and normal tissue response to irradiation in vivo: Variation with decreasing dose rates. Radiology 114:709–716, 1975.

(*16*) DEGREGORIO MW, KING OY, HOLLERAN WM, et al: Ultrafiltrate and total platinum in plasma and cerebrospinal fluid in a patient with neuroblastoma. Cancer Treat Rep 69:1441–1442, 1985.

(*17*) STEWART DJ, MIKHAEL NZ, NANJI AA, et al: Renal and hepatic concentrations of platinum: Relationship to cisplatin time, dose and nephrotoxicity. J Clin Oncol 3:1251–1256, 1985.

(*18*) STEWART DJ, BENJAMIN RS, LUNA M, et al: Human tissue distribution of platinum after *cis*-diamminedichloroplatinum. Cancer Chemother Pharmacol 10:51–54, 1982.

(*19*) Litterst CL, LeRoy AF, Guarino AM: Disposition and distribution of platinum following parenteral administration of *cis*-dichlorodiammineplatinum(II) to animals. Cancer Treat Rep 63:1485–1492, 1979.

(*20*) Douple EB, Richmond RC: Platinum complexes as radiosensitizers of hypoxic mammalian cells. Br J Cancer 37(suppl 3):98–102, 1978.

(*21*) Carde P, Laval F: Effects of *cis*-dichlorodiammineplatinum (II) and x-rays on mammalian cell survival. Int J Radiat Oncol Biol Phys 7:929–933, 1981.

(*22*) Murthy AK, Rossof AH, Anderson KM, et al: Cytotoxicity and influence on radiation dose response curve of *cis*-diamminedichloroplatinum II (*cis*-DDP). Int J Radiat Oncol Biol Phys 5:1411–1415, 1979.

(*23*) Dritschilo A, Piro AJ, Kallman AD: The effect of *cis*-platinum on the repair of radiation damage in plateau phase Chinese hamster (V-79) cells. Int J Radiat Oncol Biol Phys 5:1345–1349, 1979.

(*24*) Zwelling LA, Kohn KW: Mechanism of action of *cis*-dichlorodiammineplatinum(II). Cancer Treat Rep 63: 1439–1444, 1979.

(*25*) Zwelling LA, Anderson T, Kohn KW: DNA-protein and DNA interstrand cross-linking by *cis*- and *trans*-platinum(II) diamminedichloride in L1210 mouse leukemia cells and relation to cytotoxicity. Cancer Res 39:365–369, 1979.

(*26*) Rosenberg B: Anticancer activity of *cis*-dichlorodiammineplatinum(II) and some relevant chemistry. Cancer Treat Rep 63:1433–1438, 1979.

(*27*) Andrews PA, Wung WE, Howell SB: A high-performance liquid chromatographic assay with improved selectivity for cisplatinum and active platinum (II) complexes in plasma ultrafiltrate. Anal Biochem 143:46–56, 1984.

(*28*) Gullo JP, Litterst CL, Maguire PJ, et al: Pharmacokinetics and protein binding of *cis*-dichlorodiammineplatinum (II) administered as a four-hour or as a twenty-hour infusion. Cancer Chemother Pharmacol 5:21–26, 1980.

(*29*) Vermorken JB, van der Vijgh WJF, Klein I, et al: Pharmacokinetics of free and total platinum species after short-term infusion of cisplatin. Cancer Treat Rep 68:505–513, 1984.

(*30*) Belt RJ, Himmelstein KJ, Patton TF, et al: Pharmacokinetics of non-protein-bound platinum species following administration of *cis*-dichlorodiammineplatinum(II). Cancer Treat Rep 63:1515–1521, 1979.

(*31*) Himmelstein KJ, Patton TF, Belt RJ, et al: Clinical kinetics of intact cisplatin and some related species. Clin Pharmacol Ther 29:658–664, 1981.

Platinum Levels in Murine Tumor Following Intraperitoneal Administration of Cisplatin or Paraplatin[1]

Evan B. Douple,* Mark D. Totten, and Fred Spencer[2]

ABSTRACT—**To design protocols for exploitation of the potential for interaction among the platinum (Pt) chemotherapeutic agents cisplatin and paraplatin and radiation therapy, we measured levels of total platinum in a transplantable murine mammary adenocarcinoma (MTG-B) at different times after ip injection of platinum drugs and in various regimens. Cisplatin (20 mg/kg body weight) or paraplatin (60 or 120 mg/kg body weight) were injected ip in female C3H/HeJ mice bearing MTG-B of about 1 cm diameter. At various times after injection, tumors were removed and processed for acid digestion and total platinum analysis by atomic absorption spectrometry with a graphite furnace. The results of these experiments indicate that intratumoral Pt levels 15 minutes postinjection are higher than at 5 or 30 minutes. At 30 minutes, the Pt concentrations are of the magnitudes of 30 and 50 μM for cisplatin and paraplatin, respectively, that, when added to cells in vitro in combination with radiation therapy, produce the potentiation of cell killing. In addition, for paraplatin, intratumoral Pt levels at 30 minutes following an ip injection are approximately doubled if the tumors receive localized radiotherapy of 20 Gy immediately prior to injection of the drug. This apparent modification of paraplatin pharmacokinetics by radiotherapy might account for some of the supra-additive therapeutic potentiation in MTG-B reported for paraplatin added after irradiation.—NCI Monogr 6:129–132, 1988.**

The two Pt complexes cisplatin and paraplatin or carboplatin are currently being used in clinical treatment for a variety of tumors. The latter complex is becoming widely accepted because it has reduced toxicity while maintaining antitumor activity similar to cisplatin for certain tumors (*1,2*). Cisplatin and paraplatin have been shown to potentiate the effects of RT in mammalian cells and animal tumors when administered before or after irradiation (*3–7*). It is not surprising, therefore, that cisplatin is now being combined clinically with RT in scientists' attempts to exploit any supra-additive interactions resulting from the combined modality (*5,8*), and clinical trials have been initiated that now combine paraplatin with RT (Coughlin CT, personal communication).

Although the precise mechanisms for interactions between these 2 Pt chemotherapeutic agents and RT are not known, results of in vitro studies suggest that radiosensitization of hypoxic cells (*9–11*) and inhibition of postirradiation cellular recovery (*11–14*) might be involved. At least for these two operationally defined effects, it is reasonable for one to predict that RT should be delivered when the concentration of these drugs is maximal. We designed this experiment to measure the total Pt in a transplantable mouse mammary adenocarcinoma (MTG-B) at various times following ip injections of paraplatin to 1) compare the levels attained in tumors by paraplatin relative to cisplatin, and 2) evaluate the total Pt levels in tumors in combined modality protocols that have previously produced a suggestion of a supra-additive interaction between the 2 agents in our laboratory (*11,15,16*).

METHODS

Mouse mammary adenocarcinoma cells were transplanted sc into the flanks of 6- to 8-week-old female C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME) weighing approximately 20–25 g. The MTG-B cell suspensions were derived from tumors excised from passage mice. Tumors were minced and pressed through a Snell cytosieve, and resulting cell suspensions were centrifuged. We prepared the inocula by resuspending the known volume of packed cells in Basal Medium Eagle (GIBCO, Grand Island, NY) so as to produce a suspension for injection volumes of 0.05 ml/mouse, each containing 15% cells by volume.

The inoculation site was palpated, and diameters of the resulting tumors were measured in two perpendicular dimensions with a template. We calculated tumor volumes using the average of the two diameters to compute the volume, assuming that the tumors were spherical. We have reported the response of this tumor to either cisplatin (*16*) or paraplatin (*6,15*) in combination with RT.

Tumor-bearing mice were randomized into 6 groups; when their tumors reached an average diameter of 9.5 mm, they received injections of 60 mg paraplatin/kg body weight in Hanks' balanced salt solution (GIBCO) in a concentration of 6 mg/ml and a volume of 0.2 ml/20 g body weight. At intervals between 5 minutes and 12 hours, mice were killed by cervical luxation, and tumors were removed and frozen for storage at −20° C.

In a second experiment, mice bearing tumors of 9.5 mm diameter were randomized to 6 groups and assigned to 3 protocols. In the first protocol, mice were given ip injections of either 20 mg cisplatin/kg or 120 mg paraplatin/kg, both with 0.2 ml Hanks' balanced salt solution/20 g body

---

ABBREVIATIONS: Pt = platinum; RT = radiation therapy.

[1] Supported by Public Health Service grant CA-40500 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and Johnson Matthey, Inc., West Chester, PA.

[2] Radiobiology Laboratories, Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Hanover, NH.

* *Reprint requests:* Evan B. Douple, Ph.D., Radiobiology Laboratories, Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Hanover, NH 03756.

weight. After 25 minutes, the mice received 60 mg sodium pentobarbital/kg at a concentration of 50 mg/ml (Elkins-Sinn, Cherry Hill, NJ), and 5 minutes later, the animals were killed for Pt assay. In the second protocol, the animals were given sodium pentobarbital ip and 10 minutes later received either cisplatin or paraplatin. After another 30 minutes, the mice were killed and tumors were removed. In the third protocol, the mice received the same anesthetic, but 5 minutes later the tumors were irradiated with 20 Gy x-rays (140 kilovolt peaks, 20 milliamperes, 3 mm aluminum filtration) at a dose rate of 5.17 Gy/minute with a Maxitron-300 x-ray machine (General Electric, Milwaukee, WI). Cisplatin or paraplatin was injected immediately following the RT; mice were killed and tumors were removed 30 minutes later.

The first protocol is designed to measure Pt levels so as to suggest an appropriate time for the delivery of RT relative to the time of drug administration. With the other 2 protocols, we hoped to determine if RT has any influence on the levels of Pt attained in the tumors at 30 minutes. These latter protocols are analogous to the studies that have shown a potentiation of the effects of radiation when cisplatin or paraplatin are administered after RT. Paraplatin is less toxic compared with cisplatin and at least six times more paraplatin can be safely administered to mice on a milligram/kilogram basis. Both drugs were prepared fresh on the day of use as pure compounds (not formulated with mannitol as in a clinical preparation) and were injected as complete solutions.

After storage, frozen tumor samples were thawed and wet weights were determined for samples of approximately 100–200 mg. The samples were dried in an oven for 12 hours at 100° C and dry weights were recorded. Then they were digested in a Kjeldahl apparatus according to a modification of the method of LeRoy et al. (*17*). After the samples were heated and digests were boiled to dryness in three successive acids, nitric, followed by perchloric, and finally hydrochloric, they were resuspended in 0.1 *N* hydrochloric acid. We measured Pt in a Perkin-Elmer model 503 atomic absorption spectrometer (The Perkin-Elmer Corp., Norwalk, CT) using a model HGA-2100 graphite flameless atomizer, a system sensitive to Pt levels at about 30 parts/billion.

RESULTS

Measured total Pt levels as a function of various times following an ip injection of 60 mg paraplatin/kg body weight are illustrated in figure 1. For the estimation of concentration of Pt on a molarity basis, 1 g wet tissue was equated to 1 ml water, thereby converting micrograms/gram wet tissue into estimates of micromolar concentrations. In figure 1, it is apparent that the maximum concentration of paraplatin is reached somewhere between 5 and 30 minutes, with the concentration at 15 minutes more than twice that at 30 minutes. At 12 hours, a concentration of 11 μM total Pt was still present in the tumors.

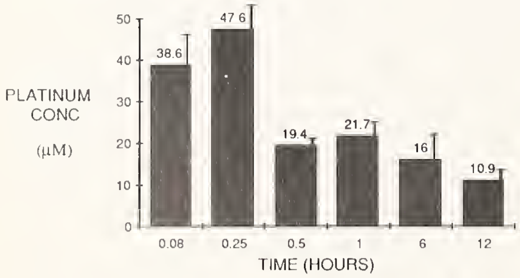


FIGURE 1.—Total Pt concentrations in MTG-B tumors as a function of time after ip injection of 60 mg paraplatin/kg body weight. *Error bars* are SEM.

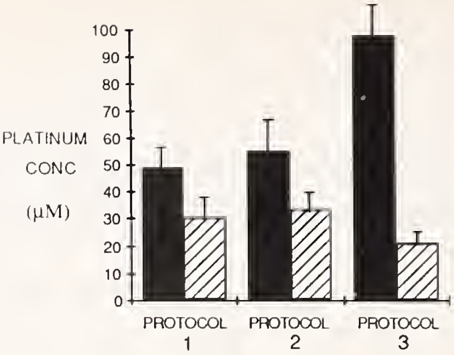


FIGURE 2.—Total Pt concentrations measured in MTG-B tumors 30 min after ip injection of 120 mg paraplatin/kg body weight (*solid columns*), or 20 mg cisplatin/kg body weight (*hatched columns*). Three protocols were used. In protocol 1, Pt injection was followed 25 min later by 60 mg sodium pentobarbital/kg. In protocol 2, the anesthetic was injected 10 min before Pt. In protocol 3, the sodium pentobarbital injection was followed 5 min later by RT of 20 Gy at 5.17 Gy/min to the tumor and injection of Pt immediately following RT.

The results of the second experiment are summarized in figure 2. At concentrations of 20 mg cisplatin/kg and 120 mg paraplatin/kg, double those used in experiment 1, the levels at 30 minutes were approximately 30 μM and 50 μM, respectively, whether the Pt preceded or followed a dose of sodium pentobarbital. However, when the mouse tumors were irradiated with 20 Gy at 5.17 Gy/minute prior to the injection of paraplatin, the concentration achieved at 30 minutes was almost twice that achieved in nonirradiated tumors.

The mean Pt concentrations and associated SEM are summarized for the 2 experiments in table 1. At 30 minutes, the level of total Pt in the tumors following paraplatin was slightly less than twice that attained following cisplatin injection, even though the total amount of Pt injected was approximately 4.6 times greater.

DISCUSSION

Although cisplatin and paraplatin have been shown to potentiate the effects of RT when administered before or immediately after RT in mammalian cells and in certain animal tumors, the optimal timing of the Pt dose relative to the RT has not been fully established. Furthermore, the clinical protocols that have been initiated to exploit the effect of the combination of Pt plus RT use a variety of drug doses and different sequencing and temporal relationships (*18*). Two mechanisms proposed to account for interactions are radiosensitization of hypoxic cells if the Pt is administered before RT (*19*), and inhibition of recovery from radiation-induced, potentially lethal damge if the drug is administered postirradiation (*12–14*). Without knowing the precise mechanism or the nature of the chemi-

Table I.—Summary of Pt concentrations

Expt No.	Protocol No.	Drug	Time postinjection, hr	No. of tumors	Mean concentration, μg/g tissue	Mean concentration, μM	SEM, μM
1		Paraplatin[a]	0.08	10	7.53	38.6	8.59
			0.25	10	9.28	47.6	6.92
			0.50	3	3.78	19.4	0.64
			1.0	6	4.23	21.7	3.20
			6.0	8	3.12	16.0	5.64
			12.0	9	2.13	10.9	2.12
2	1	Paraplatin	[b]	8	9.50	48.7	7.79
		Cisplatin		7	5.95	30.5	5.54
	2	Paraplatin		6	10.74	55.1	10.45
		Cisplatin		7	6.51	33.4	4.93
	3	Paraplatin		8	19.01	97.5	11.71
		Cisplatin		2	4.10	21.0	2.72

[a] This was the only drug used in experiment 1.

[b] Postinjection time in experiment 2 was 0.50 hr.

cal species responsible for the interaction of the 2 agents, one could reasonably propose that the greatest effect might result if the concentration of the Pt drug is at a maximum at the time of RT or immediately afterward. However, it is not known if the peak tumor concentration or a persistent bound fraction of the Pt is important for interaction with RT. The results of the first experiment in this study suggest that the maximum total Pt level attained in MTG-B is between 5 and 30 minutes and that the RT for this mouse tumor should probably be given at 15 minutes, rather than at 30 minutes as in our previous experiments (*15*). Because our earlier studies demonstrated a significant potentiation of the RT by both cisplatin and paraplatin, the results of this one predict that an even greater potentiation might be achieved if the time between Pt and RT were reduced. The Pt levels attained at 30 minutes (25 μM, for paraplatin, 15 μM for cisplatin) are of the order of those reported earlier for human tumor xenografts in immunosuppressed mice (*20,21*) and are of the magnitude required to produce a small interaction with RT, especially when cisplatin (*8*) or paraplatin (*15*) is given after RT. The levels are less than those concentrations required to produce maximum hypoxic cell radiosensitization by paraplatin that were based on in vitro studies (*11*).

The effect of the RT on the levels of total Pt 30 minutes later is especially interesting in view of the reports that paraplatin administered immediately after RT produced significant potentiation of RT (*15*). The increased levels might reflect effects on tumor blood flow with subsequent alteration of the pharmacokinetics of paraplatin. Alternatively, the effect might be mediated at the cell membrane level so as to result in more Pt entering the cell. This might explain the potentiated cell kill observed when the drug is added to cultured cells after RT. A doubling of concentration of a chemotherapeutic drug would be expected to result in a significant increase in tumor cell kill, especially if the area under the concentration-×-time curve is significantly increased. That this increase in total Pt was not evident following cisplatin administration to irradiated tumors is interesting.

These results suggest that levels of total Pt achieved in this MTG-B are of the order of magnitude required to produce modest interaction with RT. We probably should measure tumor drug concentrations and take these values into account in the design of combined modality experiments. In the case of Pt compounds, the pharmacokinetics of paraplatin is significantly different from that of cisplatin. Currently, we are determining whether the interaction between RT and paraplatin depends on the concentration of total Pt at the time of RT and immediately afterward.

REFERENCES

(*1*) Calvert AH, Harland SJ, Harrap KR, et al: JM8 development and clinical projects. *In* Platinum Coordination Complexes in Cancer Chemotherapy (Hacker MP, Douple EB, Krakoff IH, eds). Boston: Martinus Nijhoff, 1984, pp 240–252.

(*2*) Van Echo DA, Egorin MJ, Whitacre MY, et al: Phase I clinical and pharmacologic trial of carboplatin daily for 5 days. Cancer Treat Rep 68:1103–1114, 1984.

(*3*) Douple EB, Richmond RC: Interactions between platinum coordination complexes and ionizing radiation. *In* Cisplatin: Current Status and New Developments (Prestayko AW, Crooke ST, Carter SK, eds). New York: Academic Press, 1980, pp 125–147.

(*4*) Douple EB: *cis*-Diamminedichloroplatinum(II): Effects of a representative metal coordination complex on mammalian cells. *In* Cell Cycle Effects of Drugs (Dethlefsen LA, ed). Oxford: Pergamon Press, 1986, pp 215–250.

(*5*) Douple EB: The use of platinum chemotherapy to potentiate radiotherapy: Preclinical results encourage clinical trials. Platinum Metals Rev 29:118–125, 1985.

(*6*) Douple EB, Richmond RC, O'Hara JA, et al: Carboplatin as a potentiator of radiation therapy. Cancer Treat Rev 12(suppl A):111–124, 1985.

(*7*) Nias AHW: Review: Radiation and platinum drug interaction. Int J Radiat Biol 48:297–314, 1985.

(*8*) Coughlin CT, Richmond RC: Platinum based combined modality approach for locally advanced head and neck carcinomas. Int J Radiat Oncol Biol Phys 11:915–919, 1985.

(*9*) Richmond RC, Khokhar AR, Teicher BA, et al: Toxic variability and radiation sensitization by Pt(II) analogs in *Salmonella typhimurium* cells. Radiat Res 99:609–626, 1984.

(*10*) Chibber R, Sratford IJ, Ahmed I, et al: Radiosensitization of mammalian cells in vitro by transition metal complexes containing nitroimidazole ligands. Int J Radiat Oncol Biol Phys 10:1213–1215, 1984.

(*11*) O'Hara JA, Douple EB, Richmond RC: Enhancement of radiation-induced cell kill by platinum complexes (carboplatin and iproplatin) in V79 cells. Int J Radiat Oncol Biol Phys 12:1419–1422, 1986.

(*12*) Dritschilo A, Piro AJ, Kellman AD: The effect of *cis*-platinum on the repair of radiation damage in plateau phase Chinese hamster (V79) cells. Int J Radiat Oncol Biol Phys 5:1345–1349, 1979.

(*13*) Carde P, Laval F: Effects of *cis*-dichlorodiammine platinum II and x-rays on mammalian cell survival. Int J Radiat Oncol Biol Phys 7:929–933, 1981.

(*14*) Lindquist K, Douple EB, Richmond RC: Platinum drug modulation of radiation damage. Radiat Res 91:408, 1982.

(*15*) Douple EB, O'Hara JA, Jones EL: Paraplatin enhancement of radiation therapy in a murine tumor (MTG-B). *In* Anticancer Drug Research (Lapis K, Eckhardt S, eds), vol 9. Budapest: Akademiai Kiado, 1987, pp 71–80.

(*16*) Douple EB, Richmond RC: Enhancement of the potentiation of radiotherapy by platinum drugs in a mouse tumor. Int J Radiat Oncol Biol Phys 8:501–503, 1982.

(*17*) LeRoy AF, Wehling ML, Sponseller HL, et al: Analysis of platinum in biological materials by flameless atomic absorption spectrometry. Biochem Med 18:184–191, 1977.

(*18*) Schaake-Koning C, Bartelink H, Adema BH, et al: Radiotherapy and *cis*-diammine dichloroplatinum (II) as a combined treatment modality for inoperable non-small cell lung cancer: A dose finding study. Int J Radiat Oncol Biol Phys 12:379–383, 1986.

(*19*) Richmond RC, Powers EL: Radiation sensitization of bacterial spores by *cis*-dichlorodiammineplatinum(II). Radiat Res 68:20–23, 1976.

(*20*) Douple EB, Roberts JJ, Mills J, et al: Cell survival following radiation therapy (RT) and platinum chemotherapy (PT) in malignant melanoma (HX34) xenografts. Presented at the 33rd Annual Meeting of the Radiation Research Society, Los Angeles, 1985.

(*21*) Boven E, van der Vijgh WJF, Nauta MM, et al: Comparative activity distribution studies of five platinum analogues in nude mice bearing human ovarian carcinoma xenografts. Cancer Res 45:86–90, 1985.

Improvement of Differential Toxicity Between Tumor and Normal Tissues Using Intratumoral Injection With or Without a Slow-drug-release Matrix System [1]

A. C. Begg,[2,*] H. Bartelink,[2] F. A. Stewart,[2] D. M. Brown,[3] and E. E. Luck[3,4]

ABSTRACT—**The therapeutic effects of cisplatin on tumor and normal tissues were assessed when the drug was given by different administration routes either as free drug or associated with a collagen-based matrix. Tumor response was assessed by growth delay of the murine RIF1 tumor, grown subcutaneously in female C3H/km mice. Normal tissue responses were assessed by plasma clearance of [^{51}Cr]EDTA (giving an estimate of kidney damage), by the drop in peripheral white blood cells, and by a loss in mouse body weight. Intraperitoneal injections of cisplatin were the most toxic to the normal tissues for a given drug dose. Intratumoral injections of matrix-associated drug were the least toxic. Comparison of tumor growth delays for a given normal tissue damage demonstrated the superiority of all intratumoral schedules over the ip route.—NCI Monogr 6:133–136, 1988.**

The efficacy of both chemotherapy and radiosensitizers are limited by the drug concentration achievable in the tumor. This limiting concentration is set by toxicity to normal tissues (e.g., bone marrow), which determines the maximum drug dose that can be administered. This has led to investigators researching various ways of targeting drugs to tumors (*1*) to increase the exposure to the tumor relative to that of critical normal tissues. The most direct form of targeting is the injection of drug directly into a tumor, a method tried with varying degrees of success by several workers (*2–6*). Systemic exposure is often still considerable with this technique, due to rapid egress of drug from the tumor mass. In the present studies, such difficulties have prompted us to incorporate the drug into a slow-release matrix to sustain tumor drug concentrations for a longer period and to limit systemic exposure.

Our group has been particularly interested in the application of the widely used chemotherapeutic agent, cisplatin, as a radiosensitizing agent (*7–10*). This report describes our preliminary studies of combining cisplatin with a collagen-based matrix, with the eventual aim of maintaining adequately high drug concentrations in the tumor throughout a radiotherapy course of several weeks. This study concerns a comparison of the chemotherapeutic effects of cisplatin given ip or intratumorally with and without association with the slow-release matrix. To determine therapeutic benefit, we assessed both tumor response and three normal tissue end points.

MATERIALS AND METHODS

Tumor.—The RIF1 murine fibrosarcoma grown subcutaneously on the lower back of female C3H/km mice was used throughout. The tumor was maintained according to the protocol described by Twentyman et al. (*11*). Tumors for experiments were obtained by sc inoculations of 2×10^4 cells in 0.1 ml and treated when they reached approximately 8 mm mean diameter. Three perpendicular diameters of each tumor were measured with calipers approximately three times per week until the tumor exceeded 13 mm in mean diameter, at which time the mouse was killed. The growth delay for each treatment was assessed as the time to reach 2 mm above treatment size.

Kidney function.—We used the method of plasma clearance of [^{51}Cr]EDTA described by Williams and Denekamp (*12*) to assess renal damage. A single ip injection of 10 μCi [^{51}Cr]EDTA was given per mouse, and a blood sample was taken from the retro-orbital sinus 30 minutes later. The blood was centrifuged (12,000 *g*, 4 min), and 15-μl plasma samples were withdrawn and counted for ^{51}Cr radioactivity. The percent injected dose, per milliliter plasma, remaining after 30 minutes is an indication of renal function, with higher values indicating poorer function. Tests were made at 4 and 13 days after treatment and the results averaged because there was little change in response over this period (the 13-day values tended to be slightly higher than those at 4 days).

White blood cells.—Blood samples from the retro-orbital sinus of mice were taken at 4 and 13 days after treatment and diluted 1:10 or 1:20 with Turk's solution. After thorough mixing, nucleated WBC were counted with a hemocytometer.

Weight loss.—Starting on the day of treatment, all mice were weighed between three and four times per week until they were killed because of their regrowing tumor. The change from pretreatment weight was calculated on each day and averaged for each treatment group. Inspection of curves of weight change versus time showed that most groups, after an initial weight loss or halt in weight gain, eventually reached 2 g above treatment weight. Therefore, assessment of weight loss was made as the area between the weight–time curve and a horizontal line drawn at plus 2 g.

---

[1] Matrix Pharmaceutical, Inc., supplied the collagen matrix and provided partial financial support.

[2] Department of Experimental Radiotherapy, The Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Amsterdam.

[3] Matrix Pharmaceutical, Inc., Palo Alto, CA.

[4] We thank Thea Eggenhuizen for typing the manuscript.

* *Reprint requests:* A. C. Begg, Ph.D., Department of Experimental Radiotherapy, The Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

This utilized all the data and is described as "area over curve" with dimensions of grams × days.

Drug administration.—For all drug administrations, the powdered clinical formulation of cisplatin was used, which contained 9 mg NaCl and 10 mg mannitol/mg cisplatin. The collagen-based matrix was obtained as a viscous white liquid from Matrix Pharmaceutical, Inc. (Palo Alto, CA). It was mixed 1:1 with the appropriate cisplatin solution by repeated transfer between 2 syringes via a narrow-bore, stainless steel adapter. All tumors were given injections of 0.15 ml drug–matrix, giving a matrix volume:tumor volume ratio of approximately 0.5. Calculations of milligrams of drug per kilogram were based on the average weight of mice in the experiment.

The mice were anesthetized with an enflurane–oxygen mixture during the intratumoral injections. For each injection, a "fan" pattern was used that facilitated drug distribution throughout the tumor. After withdrawing the needle, some leakage of matrix out of the tumor always occurred. Leakage was assessed by the amount sucked into a 1-ml syringe without a needle. Intratumoral and ip injections of free drug were also given in 0.15 ml for direct comparison. For groups including the vasoactive agent epinephrine, the final concentration was 2 $mg \cdot kg^{-1}$.

RESULTS

We performed a pilot experiment first to test the influence of the ratio of matrix volume to tumor volume on the response of the tumor to the intratumoral injection (table 1). For both tumor sizes, the lower matrix volume gave the smaller growth delay, probably indicating a drug distribution problem. Therefore, we decided to use a matrix:tumor volume ratio of around 0.5 as a compromise between maximizing drug distribution while not causing excessive tumor disruption from mechanical damage and pressure. Further experiments were done with 0.15 ml matrix injected into 8.0- to 8.5-mm tumors (0.27–0.32 ml).

The ip injections up to 16 mg $cisplatin \cdot kg^{-1}$ were possible without deaths occurring, in contrast to previous experience with this mouse strain that indicated a maximum tolerated dose of 10 $mg \cdot kg^{-1}$ when a cisplatin solution without mannitol was used. Further experiments (data not shown) indicated that this was due partly to the mannitol and partly to the small injection volume. When the drug was given in the low injection volume of 0.15 ml, all the cisplatin could not be dissolved, necessitating injection of a saturated solution plus drug crystals. This both reduced the toxicity and made the drug less effective on the tumor (4.6 days delay compared with 8.6 days delay for 16 mg/kg).

TABLE 1.—Effect of matrix volume on RIF1 tumor response[a]

Mean tumor diameter, mm[b]	Matrix volume, ml	Matrix volume/ tumor volume	Growth delay, days[c]
7	0.06	0.33	3.2 ± 0.5
7	0.12	0.67	6.4 ± 1.8
9.5	0.06	0.13	4.3 ± 1.3
9.5	0.12	0.27	5.2 ± 0.8

[a] Drug dose was 8 mg cisplatin/kg given intratumorally; each group had 6 mice.

[b] Size is approximate; coefficient of variation = 10%.

[c] Errors are ± 1 SEM.

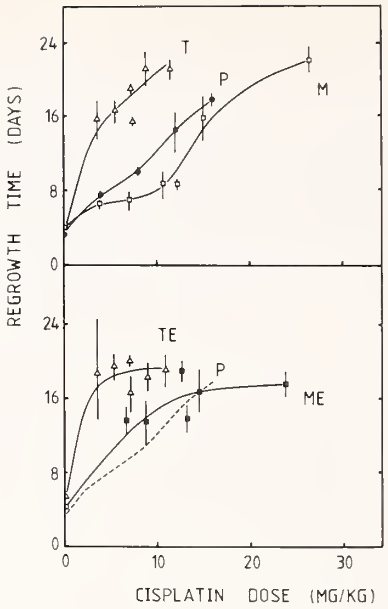


FIGURE 1.—Cisplatin dose–response curves for growth delay of subcutaneous RIF1 tumors. P: ip injection of free drug; T: intratumoral injection of free drug; M: intratumoral injection of matrix-associated drug; TE and ME are equivalent to T and M, respectively, but with the addition of epinephrine (2 mg/kg). The curve for P has been reproduced as a *dashed line* in the *lower panel.* Errors are ± 1 SEM, 5–6 mice/group. Cisplatin doses have been corrected for matrix "leakage."

Drug dose–response curves, combined from 2 experiments, for the different injection routes and formulations are shown in figure 1. The most effective treatment for a given administered dose was intratumoral injection of free drug (curve T), whereas the least effective, at low drug doses, was intratumoral injection of matrix-associated drug (curve M). Addition of epinephrine appeared to increase growth delays at low drug doses for both the free and matrix-associated drug, but had little or no effect at higher cisplatin doses (curves TE, ME). It is noteworthy that the longest observed growth delay resulted from administration of matrix-associated drug.

The results on kidney function (fig. 2) showed that the matrix-associated drug was the least toxic, made slightly

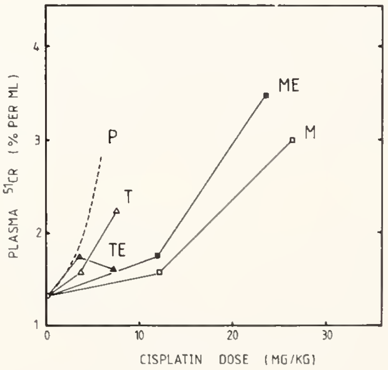


FIGURE 2.—Kidney damage assessed by plasma clearance of [^{51}Cr]EDTA after cisplatin administration by different routes and formulations. Symbols are as in fig. 1.

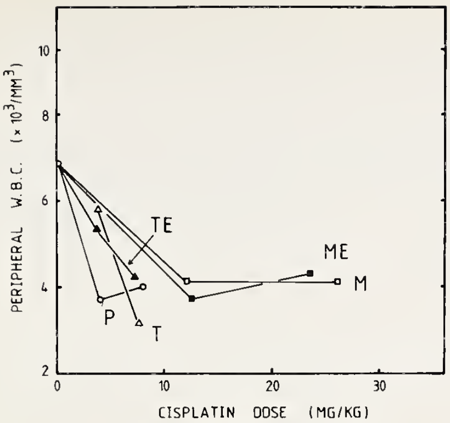


FIGURE 3.—Reduction in peripheral WBC counts by the different cisplatin treatments. Symbols are as in fig. 1.

worse by the addition of epinephrine. Free drug given intratumorally was more toxic, indicating greater systemic exposure of cisplatin, although this was still less toxic than that by ip administration.

A similar ranking order for cisplatin toxicity was seen for the reduction in peripheral WBC (fig. 3), although there appeared to be a plateau in the dose-response curve for 3 of the groups. For example, this meant that high drug doses with matrix could be given without excessive reduction in the WBC count. Again, ip injections were the most toxic.

The data on loss of body weight (fig. 4) showed that drug associated with matrix produced the least toxicity, although addition of epinephrine increased weight loss. Intratumoral injections of free drug were more toxic than with matrix-associated drug, again with the epinephrine group showing the greater weight loss, possibly the result of epinephrine alone at this relatively high dose (2 mg·kg^{-1}).

DISCUSSION

Intratumoral injection of cisplatin associated with a collagen-based matrix was less effective against the RIF1 tumor for a given drug dose than was free drug given ip or intratumorally. However, it was also the least toxic treatment assessed by all 3 normal tissue end points. As a consequence, high cisplatin doses could be administered and resulted in as long or longer growth delays than did treatments with free drug. Matrix-associated drug given intratumorally thus gave a marked therapeutic gain compared with conventional ip treatments (table 2). Injection of free drug into the tumor was also shown to be superior to that of ip injection, with or without coadministration of epinephrine. In this series of experiments, intratumoral injections with and without matrix gave approximately equal therapeutic gains.

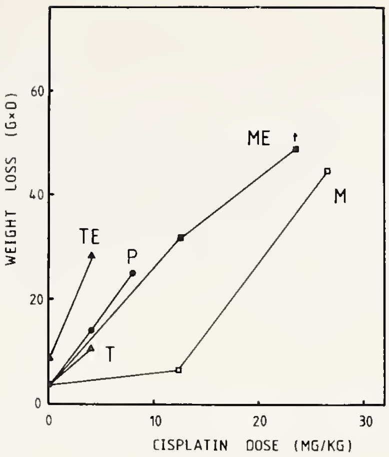


FIGURE 4.—Mouse body weight loss after different cisplatin treatments, assessed as the area between the weight loss-time curve and a line at +2 g weight gain (*see* Materials and Methods). Symbols are as in fig. 1.

In addition to cytotoxicity, radiosensitization during fractionated treatments is desirable. In preliminary studies (data not shown), we found that matrix-associated cisplatin maintained drug levels adequate for radiosensitization for longer periods than did the free drug given intratumorally. Therefore, matrix-associated cisplatin may be more suitable for radiotherapy applications.

The value of epinephrine was not proved in this study. Our rationale for its use was to take advantage of its vasoactive action to restrict blood flow locally and further limit drug outflow from the tumor. In this study, however, its addition increased toxicity in at least 2 of the 3 normal tissues. It did serve to increase tumor growth delay, but not at high doses of cisplatin, either for free or matrix-associated drug. A lower dose of epinephrine (<2 mg·kg^{-1}) may be optimal, causing less toxicity while retaining its effect on local blood flow.

One factor affecting release rates is drug solubility. For cisplatin, injections of greater than approximately 8 mg·kg^{-1} given in 0.15 ml exceeded the drug's solubility limit. Higher

TABLE 2.—Therapeutic gains: Tumor growth delay (days) from doses of cisplatin resulting in a given effect on normal tissue

Group		Kidney, 2% residual activity	Weight loss, 20 g×days	WBC, 4.5×10³/mm³
Route	Formulation			
ip	Free drug	4.0	5.1	3.0
Intratumoral	Free drug	12.6	>11.7	12.9
	Free drug+epinephrine	>13.6	11.2	13.5
	Matrix-associated drug	12.2	13.2	4.7[a]
	Matrix-associated drug+epinephrine	12.0	9.2	9.9[a]

[a] Higher drug doses (greater growth delay) are possible due to plateau in WBC dose-response curve. Other toxicity: small areas of skin ulceration were noted at high drug doses if matrix reached subcutaneous space next to tumor.

doses involved injections of a suspension comprising a cisplatin solution plus undissolved drug. This in itself may constitute a slow release system, and the contribution of such a phenomenon to the total tumor effect is now being investigated.

Our future work will be aimed at formulating and testing matrix with different drug release rates to find optimum release rates not only for cytotoxicity but also for radiosensitization in fractionated x-ray schedules. This study demonstrates the potential of intratumoral injections of cisplatin and the reduction of toxicity achievable with matrix-associated drug.

REFERENCES

(*1*) WIDDER KJ, GREEN R (eds): Drug and Enzyme Targeting. Part A. Methods in Enzymology, vol 112. London: Academic Press, 1985.

(*2*) TAKAHASHI H, NAKAZAWA S, SHIMURA T: Evaluation of postoperative intratumoral injection of bleomycin for craniopharyngioma in children. J Neurosurg 62:120–127, 1985.

(*3*) BAST RC, SEGERLING M, OHANIAN SH, et al: Regression of established tumors and induction of tumor immunity by intratumor chemotherapy. J Natl Cancer Inst 56:829–832, 1976.

(*4*) OHANIAN SH, SCHLAGER SI, GOODMAN D: Analysis of the intralesional Adriamycin-induced regression of primary and metastatic growth of line-10 guinea pig hepatoma. Cancer Immunol Immunother 8:179–187, 1980.

(*5*) MCLAUGHLIN CA, CANTRELL JL, RIBI E, et al: Intratumor chemotherapy with mitomycin C and components from mycobacterium in regression of line 10 tumor in guinea pigs. Cancer Res 38:1311–1316, 1978.

(*6*) MOTYCKA K, SLAVIK K, BALCAROVA A, et al: Treatment of solid Gardner lymphosarcoma with methotrexate sorbed on 2-hydroxyethylmethacrylate polymer in combination with leukovorin. Neoplasma 25:217–225, 1978.

(*7*) BEGG AC, VAN DER KOLK PJ, DEWIT L, et al: Radiosensitization by cisplatin of RIF1 tumour cells in vitro. Int J Radiat Biol 50:871–884, 1986.

(*8*) DEWIT L, BEGG AC, KÖHLER Y, et al: Influence of *cis*-diamminedichloroplatinum (II) on mouse duodenal crypt stem cell survival after multifraction X ray treatment. Int J Radiat Oncol Biol Phys 11:1809–1816, 1985.

(*9*) STEWART FA, BOHLKEN S, BEGG AC, et al: Renal damage in mice after treatment with cisplatinum alone or in combination with X-irradiation. Int J Radiat Oncol Biol Phys 12:927–933, 1986.

(*10*) BARTELINK H, KALLMAN RF, RAPPACCHIETTA D, et al: Therapeutic enhancement in mice by clinically relevant dose and fractionation schedules of *cis*-diamminedichloroplatinum (II) and irradiation. Radiother Oncol 6:61–74, 1986.

(*11*) TWENTYMAN PR, BROWN JM, GRAY JW, et al: A new mouse tumor model system (RIF-1) for comparison of end-point studies. JNCI 64:595–604, 1980.

(*12*) WILLIAMS MV, DENEKAMP JD: Sequential functional testing of radiation induced renal damage in the mouse. Radiat Res 94:305–317, 1983.

Response of Murine Tumors to Matrix-associated Cisplatin Intratumoral Implants

N. Y. Yu,[1,*] F. K. Conley,[2] E. E. Luck,[1] and D. M. Brown[1]

ABSTRACT—**The response to collagen matrix-associated cisplatin (*cis*-DDP-CM) implanted intratumorally into KHT and RIF-1 fibrosarcomas grown sc in C3H mice was studied. The effects on tumor growth as well as body weight and animal survival were assessed. The effect of *cis*-DDP-CM (8 mg/kg) on the growth of KHT tumor was assessed by determining the number of days required for tumors to grow to three times the pretreatment volume of 100-150 mm^3. When cisplatin (*cis*-DDP) was administered ip, the number of days required for threefold growth was 11.1 ± 2.5 SE. Administration of *cis*-DDP-CM intratumorally resulted in a value of 17.2 ± 1.7 days. Epinephrine (0.1-5.0 mg/kg) was also added to the matrix as a vasoconstrictor to further localize the activity of *cis*-DDP. This resulted in enhanced antitumor activity and, presumably, lower systemic exposure to *cis*-DDP. For *cis*-DDP administered ip, the dose required to kill 50% of the test group at 10 days after injection was approximately 14 mg/kg. When *cis*-DDP-CM was administered intratumorally in doses $\leq$30 mg/kg, no mice died. Loss in mouse body weight (>3 g) was detected with ip doses of *cis*-DDP at 8 mg/kg, but no weight loss was detected for mice treated with matrix implant delivering *cis*-DDP doses $\leq$25 mg/kg.—NCI Monogr 6:137-140, 1988.**

Luck and Brown (*1*) have demonstrated that many of the currently available anticancer drugs can be combined with specific high-molecular-weight protein carriers (matrix), so that the activity of the drug is preserved and the effect is local. Previous experience with intralesional administration of free (i.e., aqueous) drug has met with limited success in bovine squamous cell carcinoma (*2*), human warts (*3*), keratoacanthoma (*4,5*), Kaposi's sarcoma (*6*), brain tumors (*7,8*), and a number of experimental systems (*9–11*). Intralesional delivery of the free drug leads to a high local concentration for a period that varies as a function of the tissue affinity of the compound, the vascularity of the tumor, and the presence of mechanisms of inactivation (*11–14*). Moreover, plasma pharmacokinetics of intralesional delivery of free drugs demonstrate substantial systemic exposure (*15*); and since high, effective, tumor drug concentrations can only be maintained transiently, multiple treatments are usually required (*11–13*).

Matrix association of chemotherapeutic agents permits precise placement. The drug remains sequestered from systemic inactivation and a high ratio of tumor to plasma drug concentration is preserved for protracted periods, resulting in lower systemic exposure of drug to dose-limiting normal tissues (*1*). Furthermore, specific modifiers (e.g., epinephrine) may also be included in the matrix implant to further limit the diffusion of the drug away from the point of installation.

The purpose of this study was to further evaluate in vivo the use of a purified bovine collagen as an intratumoral implant matrix containing *cis*-DDP, in comparison with activity of the drug administered systemically.

A well-established cytostatic drug, *cis*-DDP has clinical activity against a wide range of human tumor histologies (*16–22*). In addition, *cis*-DDP has been shown to be a radiosensitizer in vitro and in vivo (*23–25*). The studies reported here include in vivo evaluation of *cis*-DDP–CM with RIF-1 and KHT tumors; the influence of epinephrine in the matrix; the influence of drug-matrix storage time prior to administration; and the comparison of toxicity to that produced in some normal tissues by different routes of drug administration.

MATERIALS AND METHODS

Tumor systems.—The RIF-1 and KHT fibrosarcomas were used in all experiments. They were routinely maintained by passage in vitro and in vivo as described for RIF-1 tumor by Twentyman et al. (*26*). Solid tumors were produced in 12- to 16-week-old female C3H mice (Simonson Laboratories, Gilroy, CA) by inoculating 2×10^5 cells sc into the flank. In some experiments, a second tumor was also induced on the contralateral flank. All drug treatments were carried out when the tumor size was 100–150 mm^3. For all experiments, 6–8 mice per experimental group were used.

Treatment.—All drug solutions and matrix implants were prepared immediately prior to injection except in one experiment studying the effect of storage at 4° C on *cis*-DDP bioactivity. The *cis*-DDP (Platinol, Bristol-Myers Co., Syracuse, NY), epinephrine HCl (Adrenalin HCl, 1:1,000; Parke-Davis, Morris Plains, NJ), and purified bovine CM (Collagen Corp., Palo Alto, CA), which were prepared in a sterile suspension, were admixed in appropriate concentrations so that the desired doses were delivered in injection volumes of 0.05–0.1 ml. The *cis*-DDP prepared with sterile distilled water was sonicated for ~3 minutes and was used as an aqueous suspension. The final collagen matrix concentration was 30 mg of protein/ml of drug-matrix implant. Drug-matrix implants were made by in-

---

ABBREVIATIONS: *cis*-DDP = cisplatin; CM = collagen matrix; *cis*-DDP–CM = CM-associated *cis*-DDP; 3X = three times tumor volume at initiation of treatment; LD$_{50}$ = lethal dose for 50% of test group; *cis*-DDP–CM–epi = *cis*-DDP associated with CM containing epinephrine.

[1] Matrix Pharmaceutical, Inc., Menlo Park, CA.

[2] Neurosurgical Research Laboratory, Veterans Administration Medical Center, Palo Alto, CA.

[*] *Reprint requests:* N. Y. Yu, Ph.D., Matrix Pharmaceutical, Inc., Suite 120, 1040 Marsh Rd., Menlo Park, CA 94025.

jection to the center of the tumor using a 25-gauge needle. The matrix mixture was expressed as the needle was slowly withdrawn. The needle was left in the tumor for approximately 10 seconds to limit flow back of the drug from the injection site.

Tumor growth assay.—The growth of RIF-1 and KHT tumors was determined by measurement of the treated and untreated tumors with vernier calipers three times per week. The tumor volumes were calculated from the formula:

$$V=\pi/6\times D1\times D2\times D3,$$

where D1–3 are tumor diameters measured in millimeters. The time in days required for tumors to grow to 3× was used as a measure of treatment effectiveness. All data were expressed as the mean ± SE.

Acute toxicity and body weight.—The effect of *cis*-DDP on body weight was monitored three times per week. The acute toxicity (LD_{50}) of *cis*-DDP was determined by drug dose escalation in tumor-bearing C3H mice during tumor growth studies.

RESULTS

Mice bearing 2 flank RIF-1 tumors were injected with *cis*-DDP (8 mg/kg) either as the aqueous free drug ip or intratumorally or as the CM implant intratumorally, with or without epinephrine (5 mg/kg) (table 1). The 3× tumor growth value for intratumoral injections was improved over that with systemic (ip) adminstration. The use of matrix (*cis*-DDP–CM) and matrix containing epinephrine (*cis*-DDP–CM–epi) further enhanced the antitumor effect of *cis*-DDP. The second or untreated contralateral tumor response was less affected by *cis*-DDP for these groups. Tumor growth values of ~7 days were observed for all groups inoculated intratumorally, compared with the 8.6 days observed for ip dosing. This indicates that a localization of effect had occurred with intratumoral administration. At least comparable localization and enhancement of drug effect after intratumoral injection were also observed for the KHT tumor (table 2).

The effects of the route of administration were compared for the *cis*-DDP–CM formulation versus the drug administered as the aqueous form to single KHT flank tumors. No enhancement of the antitumor effect was observed with *cis*-DDP–CM and *cis*-DDP–CM–epi delivered ip (data not shown).

TABLE 1.—Effect of CM-associated *cis*-DDP with or without epinephrine on RIF-1 tumor growth[a]

Experimental group	3× tumor growth	3× untreated contralateral tumor growth
Untreated controls	5.3 ± 0.3	5.3 ± 0.3
CM[b]	5.3 ± 0.6	4.2 ± 0.4
cis-DDP[c]	8.6 ± 1.0	8.6 ± 1.0
cis-DDP[b]	9.5 ± 0.4	7.0 ± 0.4
cis-DDP–CM[b]	10.4 ± 1.5	7.1 ± 1.1
cis-DDP–CM–epi[b]	15.6 ± 1.0	6.9 ± 0.7

[a] Values = mean No. of days ± SE. Dose of *cis*-DDP was 8 mg/kg; dose of epinephrine was 5 mg/kg.

[b] Injected intratumorally.

[c] Injected ip.

TABLE 2.—Effect of *cis*-DDP–CM with or without epinephrine on KHT tumor growth[a]

Experimental group	3× tumor growth	3× untreated contralateral tumor growth
Untreated controls	3.9 ± 0.5	3.9 ± 0.5
cis-DDP[b]	11.1 ± 2.5	11.1 ± 2.5
cis-DDP–CM[c]	17.2 ± 1.7	7.2 ± 1.2
cis-DDP–CM–epi[c]	17.2 ± 2.0	5.6 ± 0.6

[a] Values = mean No. of days ± SE. Dose of *cis*-DDP was 8 mg/kg; dose of epinephrine was 1.6 mg/kg.

[b] Injected ip.

[c] Injected intratumorally.

The influence of the epinephrine dose in *cis*-DDP–CM–epi was studied (table 3). Epinephrine doses ranged from 0.1–5.0 mg/kg in matrix containing *cis*-DDP at a dose of 6 mg/kg. No significant differences were found in this dose range since the lowest concentration of epinephrine (0.1 mg/kg) enhanced the localization and antitumor effect of *cis*-DDP as effectively as the highest epinephrine dose studied (5 mg/kg).

Finally, the effect of storage of *cis*-DDP–CM and *cis*-DDP–CM–epi at 4° C was studied (table 4). While no significant loss of activity was observed for *cis*-DDP–CM injected 30 hours after preparation, there appeared to be some loss of antitumor activity for the *cis*-DDP–CM–epi implant after the same time interval, probably due to oxidation of epinephrine.

DISCUSSION

Association of a high-molecular-weight protein carrier such as collagen with *cis*-DDP did not diminish the biologic activity of *cis*-DDP, a platinum coordination compound with activity related to the covalent binding to DNA resulting in interstrand and intrastrand cross-links (*27*). When administered intratumorally, *cis*-DDP–CM was more effective than the free (aqueous) form of *cis*-DDP injected ip (tables 1 and 2). Intratumoral administration resulted in enhancement of the antitumor effect in the treated tumor, but the results also suggested that only a limited concentration of the drug was entering the systemic circulation since antitumor effects less than those in systemically treated mice were observed in mice with untreated contralateral tumors (tables 1 and 2). Furthermore, epi-

TABLE 3.—Dose-response in KHT tumor with administration of *cis*-DDP–CM–epi[a]

Experimental group	3× tumor growth	3× untreated contralateral tumor growth
Untreated controls	6.9 ± 0.7	6.9 ± 0.7
cis-DDP–CM–epi (mg/kg)		
0	14.9 ± 3.0	11.6 ± 2.4
0.1	21.3 ± 0.9	8.6 ± 0.4
0.3	24.7 ± 3.1	8.5 ± 1.5
1.0	22.0 ± 1.5	8.4 ± 1.1
3.0	23.1 ± 1.9	7.4 ± 0.6
5.0	20.1 ± 2.7	8.0 ± 1.5

[a] Values = mean No. of days ± SE. Injection of *cis*-DDP–CM–epi was intratumoral. Dose of epinephrine was 0.1–5 mg/kg.

TABLE 4.—Effect of storage at 4° C of *cis*-DDP–CM with or without epinephrine on KHT tumor growth[a]

Experimental group	3× tumor growth	3× untreated contralateral tumor growth
Untreated controls	6.8 ± 0.4	6.8 ± 0.4
cis-DDP–CM		
3 hr	13.2 ± 1.7	8.0 ± 0.8
30 hr	13.4 ± 1.6	10.3 ± 0.8
cis-DDP–CM–epi		
3 hr	21.2 ± 1.7	7.1 ± 1.0
30 hr	16.3 ± 0.9	9.4 ± 1.0

[a] Values = mean No. of days ± SE. Injection of *cis*-DDP–CM and *cis*-DDP–CM–epi was intratumoral. Dose of *cis*-DDP–CM was 8 mg/kg; dose of epinephrine was 2 mg/kg.

nephrine used as a vasoconstrictive modifier in the matrix improved the antitumor effect of *cis*-DDP (tables 1–4). Epinephrine in the matrix, delivered intratumorally, did not show any antitumor effect by itself (data not shown). However, it significantly improved the tumoricidal effect of *cis*-DDP (tables 1–4) and may be useful with *cis*-DDP without matrix (*28*). This enhancement of *cis*-DDP activity may be due in part to the vasoconstrictive action of epinephrine temporarily interrupting the blood supply of the tumor. This would further limit the diffusion of *cis*-DDP from tumor tissue and would increase the exposure of tumor cells to the drug. The acute hypoxic condition that would result from vasoconstriction may also potentiate *cis*-DDP activity since *cis*-DDP is known to radiosensitize hypoxic cells (*29–31*) and may be more cytotoxic itself to hypoxic cells.

In addition to the improved antitumor activity of intratumorally administered *cis*-DDP–CM–epi, there was also an observed sparing of normal tissue from *cis*-DDP toxicity. For *cis*-DDP administered ip as the free drug, losses in body weight (>3 g) were observed at doses of 8 mg/kg, and the LD_{50} was ~14 mg/kg. No significant loss of body weight and no deaths of treated mice were observed for *cis*-DDP–CM–epi implants with *cis*-DDP doses ≤30 mg/kg (data not shown).

At present, local control of tumor growth is obtained primarily through surgical excision, radiotherapy, and hyperthermia. The use of cytotoxic drugs is limited to the treatment of disseminated disease, to combined modality therapy, or to a small number of drugs that can be administered to specific tissues by an intra-arterial, ip, or intracavitary route. Therapeutic drug-matrix implants present the opportunity for the administration of drugs intraoperatively, by needle through percutaneous puncture, or in combination with other therapies, while sparing the patient many of the dose-limiting side effects resulting from systemic administration.

REFERENCES

(*1*) LUCK EE, BROWN DM: The development of therapeutic drug-matrix implants: Assessment in murine tumor systems. *In* Proceedings of the Veterinary Cancer Society. Purdue University, Lafayette, IN, May 6–8, 1985. New York: Mary Ann Liebert, Inc., 1985, pp 22–23.

(*2*) BIER J, KLEINSCHUSTER S, BIER H, et al: Intratumoral immunotherapy with BCG-cell wall preparations: A new therapeutic approach for head-neck cancer. Arch Otorhinolaryngol 236:245–255, 1982 (in German).

(*3*) BUNNEY MD, NOLAN MW, BUXTON PK, et al: The treatment of resistant warts with intralesional bleomycin: A controlled clinical trial. Br J Dermatol 110:197–207, 1984.

(*4*) SAYAMA S, TAGAMI H: Treatment of keratoacanthoma with intralesional bleomycin. Br J Dermatol 109:449–452, 1983.

(*5*) ODOM RB, GOETTE DK: Treatment of keratoacanthomas with intralesional fluorouracil. Arch Dermatol 114:1779–1783, 1978.

(*6*) ODOM RB, GOETTE DK: Treatment of cutaneous Kaposi's sarcoma with intralesional vincristine. Arch Dermatol 114:1693–1694, 1978.

(*7*) TAKAHASHI H, NAKAZAWA S, SHIMURA T: Evaluation of postoperative intratumoral injection of bleomycin for craniopharyngioma in children. J Neurosurg 62:120–127, 1985.

(*8*) NAKAGAWA Y, HIRAKAWA K, UEDA S, et al: Local administration of interferon for malignant brain tumors. Cancer Treat Rep 67:833–835, 1983.

(*9*) BANKS WC, MORRIS E: Results of radiation treatment of naturally occurring animal tumors. J Am Vet Med Assoc 166:1063–1064, 1975.

(*10*) MCLAUGHLIN CA, CANTRELL JL, RIBI E, et al: Intratumor chemotherapy with mitomycin C and components from mycobacteria in regression of line 10 tumor in guinea pigs. Cancer Res 38:1311–1316, 1978.

(*11*) OHANIAN SH, SCHLAGER SI, GOODMAN D: Analysis of the intralesional Adriamycin-induced regression of primary and metastatic growth of line-10 guinea pig hepatoma. Cancer Immunol Immunother 8:179–187, 1980.

(*12*) BIER J, BENDERS P, WENZEL M, et al: Kinetics of ^{57}Co-bleomycin in mice after intravenous subcutaneous and intratumoral injection. Cancer 44:1194–1200, 1979.

(*13*) PENN RD, KROIN JD, HARRIS JE, et al: Chronic intratumoral chemotherapy of a rat tumor with cisplatin and fluorouracil. Appl Neurophysiol 46:240–244, 1983.

(*14*) SLAVIKOVA V, MOTYDKA K, SLAVIK K, et al: Distribution and pharmacokinetics of methotrexate in localized chemotherapy of solid Gardner's lymphosarcoma. Neoplasma 25:211–216, 1978.

(*15*) ENSMINGER WD, GYVES JW: Regional cancer chemotherapy. Cancer Treat Rep 68:101–115, 1984.

(*16*) EINHORN LH, DONOHUE JP: Improved chemotherapy in disseminated testicular cancer. J Urol 117:65–69, 1977.

(*17*) JACOBS C, BERTINO JR, GOFFINET DR, et al: *cis*-Platinum chemotherapy in head and neck cancers. Otorhinolaryngol 86:750–783, 1978.

(*18*) OZOLS RF, DIESSEROTH AB, JAVADPOUR N: Treatment of poor prognosis non-seminomatous testicular cancer with a "high dose" platinum combination chemotherapy regimen. Cancer 51:1803–1807, 1983.

(*19*) SOLOWAY MS, MORRIS CR, SUDDERTH B: Radiation therapy and *cis*-diammine dichloroplatinum (II) in transplantable and primary murine bladder cancer. Int J Radiat Oncol Biol Phys 5:1355–1360, 1979.

(*20*) WETLAUFER JN, FEINER AS, ROBINSON WR: Vincristine, cisplatin and bleomycin with surgery in the management of advanced metastatic nonseminomatous testis tumors. Cancer 53:502–509, 1984.

(*21*) WILLIAMS CJ, MEAD B, ARNOLD A, et al: Chemotherapy of advanced ovarian carcinoma: Initial experience using a platinum-based combination. Cancer 49:1778–1783, 1982.

(*22*) YAGODA A, WATSON RC, GONZALEZ-VITALE JC, et al: *cis*-Dichlorodiammineplatinum(II) in advanced bladder cancer. Cancer Treat Rep 60:917–923, 1976.

(*23*) CARDE P, LAVAL F: Effect of *cis*-dichlorodiammine platinum (II) and X-rays on mammalian cell survival. Int J Radiat Oncol Biol Phys 7:929–933, 1981.

(*24*) Douple EB, Richmond RC: Radiosensitization of hypoxic tumor cells by *cis*- and *trans*-dichlorodiammine platinum (II). Int J Radiat Oncol Biol Phys 5:1369–1372, 1979.

(*25*) Lelieveld P, Scoles MA, Brown JM, et al: The effect of treatment in fractionated schedules with the combination of X-irradiation and six cytostatic drugs on the RIF-1 tumor and normal mouse skin. Int J Radiat Oncol Biol Phys 11:111–121, 1985.

(*26*) Twentyman PR, Brown JM, Gray JW, et al: A new mouse tumor model system (RIF-1) for comparison of end-point studies. JNCI 64:595–604, 1980.

(*27*) Caradonna JP, Lippard SJ: Chemical and biological studies of *cis*-diamminedichloroplatinum (II) binding to DNA. *In* Platinum Coordination Complexes in Cancer Chemotherapy (Hacker MP, Douple EB, Krakoff IH, eds). Hingham, MA: Martinus Nijhoff, 1984, pp 14–26.

(*28*) Begg AC, Bartelink H, Stewart FA, et al: Improvement of differential toxicity between tumor and normal tissues using intratumoral injection with or without a slow-drug-release matrix system. NCI Monogr 6:133–136, 1988.

(*29*) Douple EB, Eaton WC, Tullont ME: Skin radiosensitization studies using combined *cis*-dichlorodiammine platinum (II) and radiation. Int J Radiat Oncol Biol Phys 5:1383–1385, 1979.

(*30*) Douple EB, Richmond RC: Platinum complexes as radiosensitizers of hypoxic mammalian cells. Cancer 37:98–102, 1978.

(*31*) Douple EB, Richmond RC: Enhancement of the potentiation of radiotherapy by platinum drugs in a mouse tumor. Int J Radiat Oncol Biol Phys 8:501–503, 1982.

Effects of Matrix-associated Chemotherapy in Combination With Irradiation In Vivo

A. E. Howes,[1,*] T. S. Herman,[1] V. P. Montoya,[1] E. E Luck,[2] and D. M. Brown[2]

ABSTRACT—Delay of tumor growth in RIF-1 fibrosarcomas in C3H mice was studied, comparing ip delivery of 5-fluorouracil (5-FU) or cisplatin (*cis*-DDP) versus collagen matrix-associated intratumoral delivery of drug with and without irradiation to a total dose of 1,500 cGy. For *cis*-DDP (6 mg/kg), the number of days required for treated tumors to attain three times their original treatment volume was 6.2 ± 1.6 SE for ip drug and 7.0 ± 1.3 for intratumoral drug matrix. The use of the vasoactive agent epinephrine (1 mg/kg) in the matrix resulted in a growth delay of 10.1 ± 2.0 days. Irradiation given 60 minutes after drug administration enhanced the delay of tumor growth to 19.2 ± 2.6 days for systemic drug and 16.7 ± 2.5 days for matrix-associated drug. The delay of tumor growth for irradiation plus matrix-associated *cis*-DDP containing epinephrine was 33.0 ± 5.4 days. X-rays alone caused a tumor growth delay of 11.2 ± 1.3 days. Similar results were found for 5-FU at a dose of 50 mg/kg, although the epinephrine in the matrix was not as effective.—NCI Monogr 6:141–143, 1988.

The ability of certain chemical drugs to enhance radiation can be demonstrated in vitro and with some mouse tumor systems in vivo. However, the potential clinical application of these strategies is generally limited by the cumulative toxicity of the drug. As cancer therapy with x-rays is fractionated (e.g., daily treatments over many weeks, requiring multiple administrations of sensitizer), an optimally high dose of chemical enhancer per fraction is generally not achievable in the clinic.

Drug-matrix implants allow administration of higher doses of sensitizer to tumors while systemic toxicity is minimized. Recently, work by Luck and Brown (*1*) and Yu et al. (*2*) has demonstrated in experimental mouse tumor systems that many of the currently available anticancer drugs as well as radiosensitizers and x-ray repair inhibitors can be successfully combined with a high-molecular-weight carrier (matrix) so that the activity of the drug is preserved and the effect is local. These therapeutic drug-matrix implants allow for precise placement in tumors by injection of a nonmigrating, biologically tolerated, drug-secreting mass. This provides a high local sustained-release drug concentration in the tumor, with limited diffusion into the systemic circulation (*1*). The therapeutic drug-matrix implant may be compared conceptually with interstitial brachytherapy, in which radioactive sources such as ^{192}Ir or ^{125}I are placed in tumors for continuous delivery of radiation to a precise volume.

It is the purpose of this study to evaluate in vivo the use of 5-FU (*3–5*) or *cis*-DDP (*6–8*) matrix implants in combination with radiation. The effect on injection time prior to irradiation as well as the use of the vasoconstrictor epinephrine in the matrix was studied.

MATERIALS AND METHODS

Tumor system.—The RIF-1 fibrosarcoma was used in these experiments. It was routinely maintained by passage in vitro and in vivo as described by Twentyman et al. (*9*). Solid tumors were produced in 12- to 16-week-old C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME) by inoculating 2×10^5 cells sc into the right rear leg. All drug and radiation experiments were carried out when the tumor size was 100–125 mm^3. For all experiments, 6 mice per experimental group were used.

Drug treatments.—All drug solutions and matrix implants were prepared immediately prior to injection. The *cis*-DDP (Platinol, Bristol-Myers Co., Syracuse, NY), 5-FU (Hoffmann-La Roche, Inc., Nutley, NJ), epinephrine HCl (Adrenalin HCl, 1:1,000; Parke-Davis, Morris Plains, NJ), and purified bovine CM (Collagen Corp., Palo Alto, CA) were admixed in appropriate concentrations so that the desired doses were delivered in injection volumes of 0.05–0.1 ml. The *cis*-DDP, which was prepared with sterile distilled water, was sonicated for ~3 minutes and was used as an aqueous suspension. The final CM concentration was 30 mg of protein/ml of drug-matrix implant. The drug-matrix implant was injected through a 25-gauge needle into the center of the tumor as the needle was slowly removed.

X-ray treatment.—Tumor-bearing mice were irradiated in lead jigs exposing only the tumor-bearing leg with a ^{137}Cs source (Gammacell 40, Atomic Energy of Canada, Ltd., Ottawa, Ontario) at a dose rate of 100 cGy/minute.

Tumor growth delay assay.—The growth delay of RIF-1 tumors was determined by measurement of the treated and untreated tumors with vernier calipers three times per week. The tumor volumes were calculated from the formula:

$$V = \pi/6 \times D1 \times D2 \times D3,$$

where D1–3 are tumor diameters measured in millimeters.

The time in days required for the tumors to grow to three

ABBREVIATIONS: 5-FU = 5-fluorouracil; *cis*-DDP = cisplatin; CM = collagen matrix; *cis*-DDP–CM = CM-associated *cis*-DDP; *cis*-DDP–CM–epi = *cis*-DDP associated with CM containing epinephrine; CM–epi = CM containing epinephrine; 5-FU–CM = CM-associated 5-FU; 5-FU–CM–epi = 5-FU associated with CM containing epinephrine.

---

[1] Division of Cancer Biology, Joint Center for Radiation Therapy, Harvard Medical School, Boston, MA.

[2] Matrix Pharmaceutical, Inc., Menlo Park, CA.

[*] *Reprint requests:* A. E. Howes, M. D., Division of Cancer Biology, Joint Center for Radiation Therapy, Harvard Medical School, 50 Binney St., Boston, MA 02115.

times their original treatment volume was used as a measure of treatment effectiveness. Mice with no evidence of disease at 60 days were considered to be cured. All data are expressed as the mean ± SE.

RESULTS

The RIF-1 tumor-bearing mice were injected ip with *cis*-DDP at a dose of 6 mg/kg alone or either 30 or 60 minutes prior to irradiation to a total dose of 1,500 cGy. The *cis*-DDP–CM was administered at a dose of 6 mg/kg with or without epinephrine at a dose of 1 mg/kg. It was injected intratumorally alone or prior to irradiation at the same time intervals. The results are summarized in table 1. The tumors of mice treated with drug ip were not significantly affected by *cis*-DDP alone at this dose. Slight enhancement of the tumor response (i.e., increased growth delay values) was observed for *cis*-DDP–CM–epi (10.1 vs. 6.2 days). Furthermore, the CM–epi alone had no effect on tumor growth delay. With irradiation to 1,500 cGy at 30 minutes after drug injection, the response to radiation and drug was less than additive for all experimental groups. However, when the interval was increased to 60 minutes between *cis*-DDP administration and irradiation, we observed additive growth delays for groups treated with *cis*-DDP ip or *cis*-DDP–CM intratumorally. Furthermore, in the group treated with *cis*-DDP–CM–epi intratumorally plus x-rays, the growth delay was 33 days, which is greater than additive in this single-dose experiment.

The RIF-1 tumor was unresponsive to 5-FU at a dose of 50 mg/kg administered either ip or as the CM implant intratumorally (table 2). The growth delay was approximately the same as in the untreated control tumors. The irradiation of the tumors of all 5-FU-treated mice resulted in enhancement of the antitumor effects, which are greater than additive. A 60-minute time interval between drug administration and irradiation was superior to 30 minutes for all groups. Administration of 5-FU–CM intratumorally resulted in the greatest response, with 2 of 6 mice without measurable tumors at 60 days after treatment.

TABLE 1.—Effect of CM-mediated delivery of *cis*-DDP in combination with x-rays on RIF-1 tumor growth[a]

Experimental group (6 mice/group)	3× tumor growth (Mean No. of days ± SE)[b]	Added delay (No. of days)
Untreated controls	5.4 ± 0.9	—
X-rays alone	11.2 ± 1.3	5.8
cis-DDP[c]	6.2 ± 1.6	0.8
cis-DDP–CM[d]	7.0 ± 1.3	1.6
cis-DDP–CM–epi[d]	10.1 ± 2.0	4.7
CM–epi[d]	3.3 ± 1.5	−2.1
30 min before x-rays		
cis-DDP[c]	10.1 ± 0.7	4.7
cis-DDP–CM[d]	9.8 ± 1.8	4.4
cis-DDP–CM–epi[d]	14.5 ± 2.3	9.1
CM–epi[d]	12.9 ± 2.2	7.5
60 min before x-rays		
cis-DDP[c]	19.2 ± 2.6	13.8
cis-DDP–CM[d]	16.7 ± 2.5	11.3
cis-DDP–CM–epi[d]	33.0 ± 5.4	27.6
CM–epi[d]	7.7 ± 1.1	2.3

[a] Dose of *cis*-DDP was 6 mg/kg; x-ray dose was 1,500 cGy.
[b] 3× = 3 times tumor volume at initiation of treatment.
[c] Injected ip.
[d] Injected intratumorally.

TABLE 2.—Effect of CM-mediated delivery of 5-FU in combination with x-rays on RIF-1 tumor growth[a]

Experimental group (6 mice/group)	3× tumor growth (Mean No. of days ± SE)	Added delay (No. of days)
Untreated controls	3.1 ± 0.6	—
X-rays alone	8.2 ± 1.1	5.1
5-FU[b]	3.1 ± 0.5	0
5-FU–CM[c]	2.4 ± 0.1	−0.7
5-FU–CM–epi[c]	4.3 ± 1.7	1.2
CM–epi[c]	3.5 ± 1.2	0.4
30 min before x-rays		
5-FU[b]	17.3 ± 4.8	14.2
5-FU–CM[c]	21.4 ± 2.4	18.3
5-FU–CM–epi[c]	16.1 ± 3.6	13.0
CM–epi[c]	4.8 ± 0.3	1.7
60 min before x-rays		
5-FU[b]	20.6 ± 1.4	17.5
5-FU–CM[c]	>26.0[d]	>22.9
5-FU–CM–epi[c]	15.0 ± 5.1	11.9
CM–epi[c]	6.3 ± 1.4	3.2

[a] Dose of 5-FU was 50 mg/kg; x-ray dose was 1,500 cGy.
[b] Injected ip.
[c] Injected intratumorally.
[d] Two of 6 mice had no evidence of disease at day 60.

DISCUSSION

In these experiments, the time interval between drug administration and irradiation was found to be very important for maximum treatment effectiveness. It was observed that a 60-minute interval gave superior results for both drugs. However, the optimal time for drug administration is not known as yet. Preliminary time course experiments for *cis*-DDP–CM given intratumorally versus *cis*-DDP ip suggest that between 60 and 90 minutes before irradiation may be optimal (data not shown).

The use of epinephrine in the matrix as a vasoconstrictive agent to further localize the drugs within the tumor enhanced the antitumor effect of *cis*-DDP (*cis*-DDP–CM–epi) administered intratumorally either alone or in combination with radiation (table 1). Radioresistance, however, was observed for the groups treated with matrix containing CM and epinephrine alone (tables 1 and 2); this was expressed as growth delay values lower than those for the controls that received radiation alone. Furthermore, results in tumors treated with 5-FU–CM–epi plus radiation were poorer than in those treated with 5-FU–CM plus radiation, suggesting that the vasoconstrictor epinephrine induced an acute radioprotective hypoxia. Interestingly, *cis*-DDP–CM–epi plus radiation was more effective than either *cis*-DDP or cis-DDP–CM plus radiation.

The data from these initial experiments demonstrate the feasibility and potential effectiveness of the combination of drug-matrix implants with radiation for the treatment of localized disease. The matrix-associated drug in these experiments was more effective as a sensitizer than the drug delivered systemically, presumably without extensive diffusion into the systemic circulation (*1,2*).

In the treatment of cancer, the therapist must overcome two major problems: local control of the primary neoplasm and eradication of metastatic disease. Recent assessment indicates that, despite advances in therapy, approximately 30%–40% of all patients die as a result of uncontrolled local disease (*10*). Consequently, a new strategy that will improve local control should significantly impact on both the duration and the quality of survival. Drug-matrix implants containing 5-FU and *cis*-DDP in conjunction with x-rays may be of important benefit when local tumor control is a concern. Matrix implants may offer the oncologist the opportunity to use a range of cytotoxic and radiation-sensitizing agents for the treatment of neoplasms that could not be tolerated if administered systemically. Furthermore, a number of new treatment strategies could develop including preradiotherapy or postradiotherapy drug-matrix implants or the local use of x-ray repair inhibitors. Finally, drug-matrix implants may be useful in enhancing tumor cell killing in such radioresistant tumors as osteogenic sarcoma and melanoma.

REFERENCES

(*1*) Luck EE, Brown DM: The development of therapeutic drug-matrix implants: Assessment in murine tumor systems. *In* Proceedings of The Veterinary Cancer Society, Purdue University, Lafayette, IN, May 6–8, 1985. New York: Mary Ann Liebert, Inc., 1985, pp 22–23.

(*2*) Yu NY, Conley FK, Luck EE, et al: Response of murine tumors to matrix-associated cisplatin intratumoral implants. NCI Monogr 6:137–140, 1988.

(*3*) Thomas G, Dembo A, Beale F, et al: Concurrent radiation, mitomycin and 5-fluorouracil in poor prognosis carcinoma of cervix: Preliminary results of a phase I–II study. Int J Radiat Oncol Biol Phys 10:1785–1790, 1984.

(*4*) Byfield JF, Barone R, Mendelsohn J, et al: Infusional 5-fluorouracil and x-ray therapy for non-resectable esophageal cancer. Cancer 45:703–708, 1980.

(*5*) Byfield JE, Calabro-Jones P, Klisak I, et al: Pharmacologic requirements for obtaining sensitization of human tumor cells in vitro to combined 5-fluorouracil or ftorafur and x-rays. Int J Radiat Oncol Biol Phys 8:1923–1933, 1982.

(*6*) Douple EB, Richmond RC: Platinum complexes as radiosensitizers of hypoxic mammalian cells. Br J Cancer (suppl III)98:98–102, 1978.

(*7*) Carde P, Laval F: Effect of *cis*-dichlorodiammine platinum (II) and x-rays on mammalian cell survival. Int J Radiat Oncol Biol Phys 7:929–933, 1981.

(*8*) O'Hara J, Douple EB, Richmond RC: Enhancement of radiation-induced cell kill by platinum complexes (carboplatin and iproplatin) in V79 cells. Int J Radiat Oncol Biol Phys 12:1419–1422, 1986.

(*9*) Twentyman PR, Brown JM, Gray JW, et al: A new mouse tumor model system (RIF-1) for comparison of end point studies. JNCI 64:595–604, 1980.

(*10*) Suit HD: Statement of the problem pertaining to the effect of dose fractionation and total treatment time on response of tissue to x-irradiation. *In* Time and Dose Relationship in Radiation Biology as Applied to Radiotherapy. Brookhaven National Laboratory Publications, No. 50203-C-57. Brookhaven, NY: Brookhaven National Laboratory, pp VII–X, 1969.

Administration of Radiation or Cyclophosphamide Versus Alternated Radiation and Cyclophosphamide Treatment of Primary Tumor and Pulmonary Metastases[1]

William B. Looney* and Harold A. Hopkins[2]

ABSTRACT—**The experimental tumor 3924A in ACI rats metastasizes to the lungs, but this is rarely apparent unless the life span of the animal is increased by treatment of the tumor. Pulmonary metastases were prevalent among animals given radiation daily or in multiple fractions per day over periods of 3-4 weeks at total doses ranging from 3,000 to 11,250 rad. Multiple doses of cyclophosphamide, each at the level that was lethal to 10% of the animals, did not cure the primary tumor but appeared to delay the appearance of metastases. Treatment schedules that alternated the delivery of multiple 250-rad fractions of radiation during 2-day periods with cyclophosphamide at a dose of 150 mg/kg for a total of three courses were effective in curing the primary tumor and eliminating pulmonary metastases.—NCI Monogr 6:145-146, 1988.**

The spread of malignant tumor cells from the primary tumor to form metastases at different sites is a major cause of death in cancer patients. This report summarizes the results of studies on the effects of radiotherapy and chemotherapy on an experimental solid tumor. A majority of these primary tumors can be cured, and the pulmonary metastases can be virtually eliminated by a new, more effective utilization of these two treatment modalities.

MATERIALS AND METHODS

The tumors were obtained by injecting 3924A tumor cells into the flank of ACI inbred rats so that the primary tumor could be irradiated with minimal radiation to the host, which was accomplished by shielding. Viable tumor tissue was minced with scissors and loaded into 1-ml syringes; 0.1 ml containing $\sim 2 \times 10^7$ cells was delivered sc through a 14-gauge needle.

Tumors were irradiated with a 250-kV 15-mA GE Maximar 250-III with a half-value layer of 1.39 mm Cu at a rate of 280 rad/minute. Filtration was performed through 0.5 mm Cu and 1.0 mm Al. Before irradiation, animals were anesthetized with ether and placed in a lead shield through which the tumor protruded. The CP, which was supplied by Mead Johnson Research Center (Evansville, IN), was dissolved in 0.9% NaCl solution and given by ip injection. Treatment of pulmonary metastases was confined to CP, while the primary tumor received both CP and radiation.

Treatments were initiated when the tumor volume was approximately 250 mm^3. The T 40 V_0 for individual tumors was determined by interpolation between measurements; median times to T 40 V_0 were determined from Kaplan-Meier plots.

The complete lung was removed and examined visually for large metastases, then observed under a Bausch & Lomb magnifier with a magnifying factor of approximately 1.5. Cross sections were made at 1- to 2-mm intervals in the lobes of both lungs and were visually examined for internal pulmonary metastases. The metastases are much lighter in color than the lung and can be easily detected by visual examination with a magnifying glass. The smallest metastasis that can be observed with the magnifier is about 0.5–1.0 mm in diameter. Karyotypic studies showed that metastases were derived from the primary tumor.

RESULTS

Radiation Alone

Pulmonary metastases were prevalent in the groups of animals given daily radiation doses of 100–375 rad and total doses of 3,000–11,250 rad over a 1-month period (table 1). Pulmonary metastases were also prevalent when the total radiation dose to the primary tumor was 4,500–9,000 rad over a 23-day period and the radiation was given in MFD. The primary tumors were not cured by radiation given in daily fractions or in MFD at the largest total doses used; i.e., 11,250 and 9,000 rad, respectively (*1,2*). Large single doses of radiation (4,500–6,000 rad) to the primary tumor gave cure rates of 88%–100%, and at autopsy 116–120 days later, pulmonary metastases had been virtually eliminated.

Cyclophosphamide Alone

Five groups of animals were given CP in single doses of 50–250 mg/kg. There were no pulmonary metastases in the group given CP at a dose of 50 mg/kg; however, the median survival was only 40 days. The number of animals with pulmonary metastases per 10-animal group increased from 1 to 3 as the CP dose was increased from 100 to 250 mg/kg. However, the median survival increased from 45 to

---

ABBREVIATIONS: CP = cyclophosphamide; T 40 V_0 = time required for tumor(s) to reach 40 times initial volume; MFD = multiple fractions per day.

[1] Supported in part by Public Health Service grants CA-20516 and CA-35372 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Division of Radiobiology and Biophysics, University of Virginia School of Medicine, Charlottesville.

* *Reprint requests:* William B. Looney, M.D., Division of Radiobiology and Biophysics, Box 392, University of Virginia Hospital, Charlottesville, VA 22908.

TABLE 1.—Growth delay, median survival, and incidence of pulmonary metastases with increasing daily radiation fraction size[a]

Radiation (rad)	Total dose (rad)	Complete response[b]	Tumor cure	Median time to T 40 V_0 (days)	Median survival (days)	Incidence of metastases ($n=10$/group)
100×10	3,000	0	0	24	48	50%
150×10	4,500	0	0	52	76	60%
188×10	5,640	0	0	62	85	80%
250×10	7,500	0	0	82	101	90%
375×10	11,250	0	0	140	>155[c]	50%

[a] Radiation was given on days 0–9, 11–20, and 22–31.

[b] No tumors were found at autopsy.

[c] Day rats were killed.

TABLE 2.—Tumor cure, median survival, and incidence of pulmonary metastases with increasing No. of 250-rad radiation fractions combined with CP with 7-day interval between modalities[a]

Treatment						
Radiation (rad)	CP (mg/kg)	Total dose (rad)	Complete response[b]	Tumor cure	Median survival	Incidence of metastases ($n=10$/group)
250×3	150	4,500	0	0	161	50%
250×4	150	6,000	1	1	>171[c]	20%
250×5	150	7,500	7	7	>172[c]	0%
250×6	150	9,000	6	6	>172[c]	10%
250×7	150	10,500	5	5	117	0%

[a] Radiation was given on days 0 and 1, 14 and 15, and 28 and 29; CP was given on days 7, 21, and 35.

[b] No clinically detectable evidence of tumor.

[c] Day rats were killed.

55 days, allowing a longer time interval for the metastases to become visible. No primary tumors were cured with CP alone at any dose. No pulmonary metastases occurred when two, three, four, or five doses of CP (150 mg/kg) were given every 11 days, with the exception that 3 of 10 animals given four doses of CP survived >90 days after initial treatment, and these animals had metastases at autopsy. The greatest median survival (70 days) occurred with three doses of CP (each 150 mg/kg), and no metastases were observed in this group. It appears that CP delays the appearance of metastases in rats not otherwise cured of the primary tumor.

Radiation Alternated With Cyclophosphamide

The incidence of metastases was observed for treatment schedules in which MFD irradiation was alternated with CP. The radiation was given as multiple 250-rad fractions during 2-day periods, repeated at intervals of 11 or 14 days. A total of three doses of CP (each 150 mg/kg) were given at intervals of 11 or 14 days, either 1 day or 7 days after completion of each course of MFD irradiation. Low total doses of MFD irradiation alternated with CP (750–3,000 rad) resulted in 1–8 pulmonary metastases per group of 10 rats and no cures of the primary tumor (*1,2*). Only 1 metastasis occurred at doses of 4,500–7,500 rad in the 3 groups given CP 1 day after each of three courses of MFD irradiation; in the 3 groups, cures of primary tumors were observed in 3, 5, and 6 animals. Increasing the time between radiation and CP delivery from 1 to 7 days and increasing the overall time of the three courses of radiation alternated with CP from 24 to 35 days resulted in 5 metastases at 4,500 rad and 2 at 6,000 rad but none at doses of 7,500–10,500 rad, with 1 exception in the 9,000-rad group. The primary tumor cure rates were 70%, 60%, and 50% for total radiation doses of 7,500, 9,000, and 10,500 rad, respectively (table 2).

REFERENCES

(*1*) LOONEY WB, HOPKINS HA, CARTER WH JR: Solid tumor models for the assessment of different treatment modalities. XXII. The alternate utilization of radiotherapy and chemotherapy. Cancer 54:416–425, 1984.

(*2*) LOONEY WB, HOPKINS HA, CARTER WH JR: Solid tumor models for the assessment of different treatment modalities. XXIII. A new approach to the more effective utilization of radiotherapy alternated with chemotherapy. Int J Radiat Oncol Biol Phys 11:2105–2117, 1985.

Systematic Investigation Into Interaction of Ionizing Radiation and Doxorubicin in the Dunning R3327G Prostatic Adenocarcinoma Model [1]

Charles F. Gottlieb,[2,*] G. Burton Seibert,[3] and Norman L. Block[2,4]

ABSTRACT—**The interaction of doxorubicin and radiation has been systematically studied in the Dunning R3327G prostatic adenocarcinoma, the preeminent animal model for human prostatic cancer. Subcutaneous tumors (produced by injection of 10^7 cells) were treated when about 1 cm^3 in volume (19–22 days postimplant). Each modality was used at 1 of 3 dose levels; 2, 4, and 9 mg/kg for doxorubicin; and 5, 15, and 25 Gy for radiation. Single treatment with each agent was combined, in both sequences and five delay times (0.5, 12, 24, 48, and 120 hr) between agents. Growth of individual tumors was fit to a quadratic exponential growth model which was solved for the growth delay and growth rate at twice initial volume. Analysis of variance identified significant interactions for doxorubicin and radiation (due to drug toxicity), sequence and delay, and sequence and radiation, in addition to the four factors individually. The effect on the tumor of combined doxorubicin and radiation is basically additive. Sequence and delay are important in overall tumor control.—NCI Monogr 6:147–153, 1988.**

The Dunning prostatic adenocarcinoma is the preeminent tumor model for the human prostatic tumor, which is the second leading cause of cancer death in elderly men. With regard to hormonal, chemotherapeutic, and radiotherapeutic manipulations, the similarities of the model have been widely documented (*1–8*), and it appears to be the best system for studying prostate cancer (*9,10*).

Combined modality cancer therapy has its origin in the failures of individual treatment modalities to control the disease (*11,12*). The principles of multimodality therapy may be adapted from those expounded by Krakoff (*13*) as follows: 1) agents that are active when used alone, 2) agents that have a biochemical basis for suspect synergy, 3) agents with different mechanisms of action, and 4) agents that produce nonadditive toxicities. These principles have been specifically applied to drugs and radiation in a recent review by Bellamy and Hill (*14*).

Combined doxorubicin and radiation have been examined in a number of studies, both experimental (*14*) and clinical. Potential synergism can result from the different cell-cycle phase specificities of the two as radiation preferentially kills in G_2/M, whereas doxorubicin acts in S phase (Gottlieb CF, Pollack A, Ciancio G, et al, submitted for publication). The issues of timing and sequence of agent application are of potential importance and are the primary thrust of this communication. Investigators conducting previous studies with doxorubicin and radiation, using systems other than the Dunning tumor, have generally concentrated on closely sequenced administration and, therefore, may have failed to identify patterns manifesting one or more cell generations after treatment. In fact, for the R3327 tumor, the accumulation of cells blocked by doxorubicin in G_2 does not peak until 4 days after drug treatment and persists for more than 2 weeks (Gottlieb CF, Pollack A, Ciancio G, et al, submitted for publication).

MATERIALS AND METHODS

Animals.—The rats used in this study were (F344× COP)F_1 males reared in our breeding colony under conventional animal husbandry conditions. The animals were 3 to 4 months of age when implanted with tumor.

Tumor.—The rapidly growing, hormonally sensitive R3327G prostatic adenocarcinoma has been maintained by serial transplantation. The procedures of cell dispersion and sc inoculation have been described in detail (*4*). Briefly, tumors selected for transplant are minced and enzymatically dispersed. Washed and resuspended cells are inoculated sc into experimental animals at 10^7 cells/rat.

A common pool of tumor cells cryopreserved at the 24th in vivo transplant generation was used for this study; cells from this pool were inoculated into carrier rats. These tumors provided cells for implantation of the experimental tumors (26th generation) used in the reported study. We developed this approach, using a tumor cell pool, to minimize the biologic variation potentially produced by implanting the tumor in animals at different times.

Doxorubicin.—Doxorubicin, purchased as Adriamycin from Adria Laboratories (Columbus, OH), was reconstituted and diluted with saline. One of three drug doses, i.e., 2, 4, or 9 mg/kg, was administered ip in a 1-ml volume at 19–24 days after tumor implantation.

Radiation.—Cobalt-60 radiation was from a Gammabeam 150, Atomic Energy of Canada, Ltd. (Ottawa, Ontario, Canada). This panoramic industrial irradiator was collimated for tumor exposure with custom ports made from lead bricks. Each port consists of a 2.5-cm cylindrical hole through 10-cm thick lead. A total of three ports

---

[1] Supported in part by Veterans Administration Merit Review No. 0998-005 and the Weeks Endowment Fund.

[2] Departments of Radiation Oncology (*C. F. Gottlieb*) and Urology (*N. L. Block*), University of Miami School of Medicine, FL.

[3] Medical Computing Resources Center, University of Texas Health Science Center at Dallas.

[4] Veterans Administration Hospital, Miami, FL.

* *Reprint requests:* Charles F. Gottlieb, Ph.D., Department of Radiation Oncology (D31), University of Miami School of Medicine, P.O. Box 016960, Miami, FL 33101.

Table 1.—Factorial design for study of interaction of doxorubicin and radiation

Factor	Level description[a]	No. of levels
Doxorubicin	2, 4, 9 mg/kg	3
Radiation	5, 15, 25 Gy	3
Sequence	Doxorubicin + radiation, radiation + doxorubicin	2
Delay	0.5, 12, 24, 48, 120 hr	5
Replication		5

[a] Each level of each factor was tested in combination with every level of every other factor for a total of 90 (3×3×2×5) distinct treatments. With 5 replicates, 450 tumor-bearing rats were required.

allows simultaneous treatment of 3 tumors. Thick plastic (0.5 cm) placed at the tumor (between it and the radiation source) served both to position the tumor and to assure buildup of electronic equilibrium and proper tumor dose. Dosimetric calibration was made with a Baldwin-Farmer dosimeter calibrated by the National Bureau of Standards. Dose uniformity across the tumor was approximately 10% (or better) of the stated dose. The dose rate was 1.8–1.9 Gy/minute. Animals were immobilized for treatment with 54 mg ketamine/kg, i.e., Vetalar from Parke-Davis/Warner-Lambert (Morris Plains, NJ) and 0.55 mg acepromazine/kg, i.e., Acepromazine Maleate from Ayerst Laboratories, Inc. (New York, NY) given im.

Assay of tumor growth.—Growth of the tumors was monitored over time after treatment by three-axis caliper measurements corrected for ellipsoid shape [length × width × height × 0.5236; (*15*)]. Depending on the time rate of change in tumor size, the measurement interval was twice weekly, weekly, or once every other week.

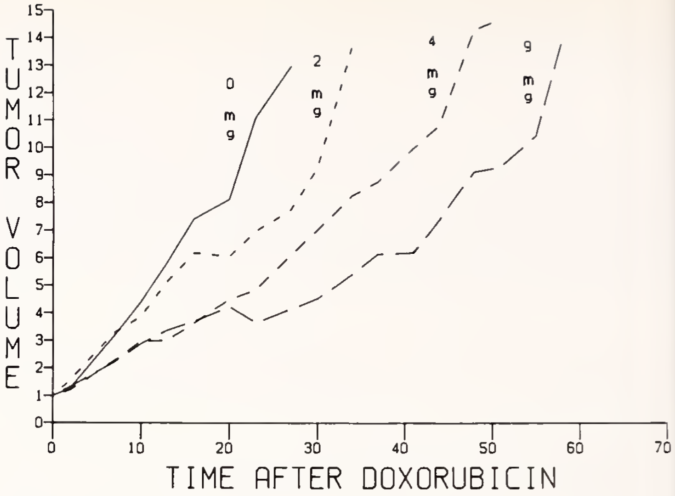


Figure 1.—Tumor growth following a single treatment with doxorubicin at 0, 2, 4, or 9 mg/kg. Time is in days after drug treatment. Each curve represents an average of tumor volumes previously normalized to unity at the time of treatment. Animals whose tumors become grossly necrotic and hemorrhagic undergo spontaneous debulking; these tumors were excluded from the average for times after this had occurred. The curves initially represent 10 animals and have been terminated at the point having fewer than 4 tumors to average. Average initial tumor volumes (cubic centimeter) were control, 2.04; 2 mg/kg, 1.48; 4 mg/kg, 1.61; and 9 mg/kg, 1.12.

Experimental design.—A factorial design was used for this study and is outlined in table 1. The choices of dose level for doxorubicin were 2, 4, and 9 mg/kg and for radiation 5, 15, and 25 Gy, and they were derived from the corresponding single agent dose versus response studies. The doses were selected on the following basis: The "high" dose is that dose producing considerable reduction in tumor growth with acceptable morbidity/mortality. The "low" dose is that dose producing a minimal, although measurable, reduction in tumor growth. The "medium" dose is a mid-range dose approximately halfway between the low and high doses. Both sequences (doxorubicin followed by radiation and radiation followed by doxorubicin) were studied, as well as five delay times between treatments with the first and second agents. We selected the treatment delay times, 0.5, 12, 24, 48, and 120 hours, to allow detection of potential synergism regardless of the mechanism of interaction.

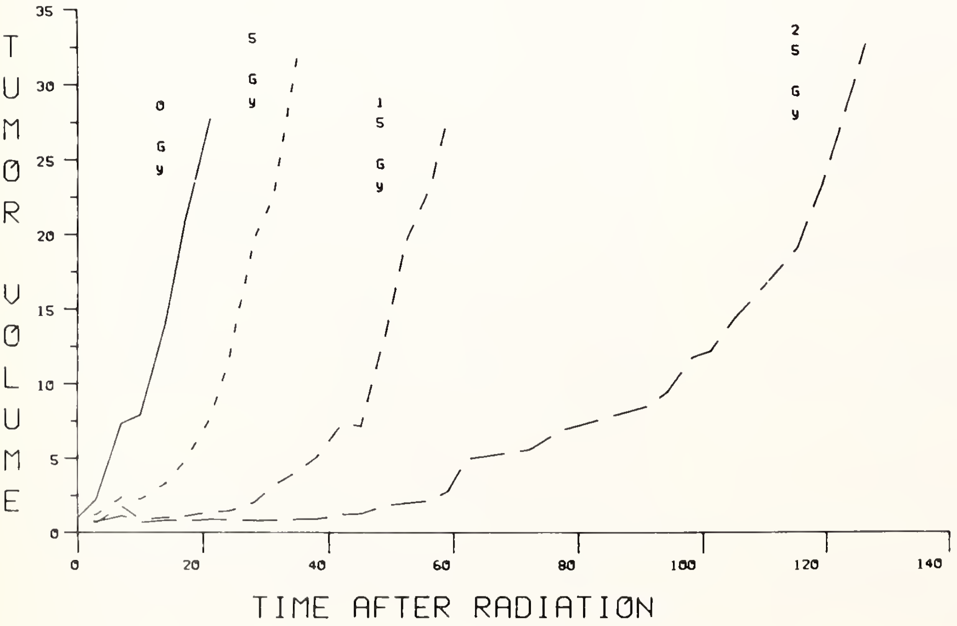


Figure 2.—Tumor growth following a single treatment with radiation at 0, 5, 15, or 25 Gy. Time is in days after radiation treatment. *See* fig. 1 legend for description of how curves were produced. Average initial tumor volumes (cubic centimeter) were control, 0.70; 5 Gy, 1.44; 15 Gy, 0.83; and 25 Gy, 0.80.

TABLE 2.—Tumor growth following treatment by radiation only[a]

Dose, Gy	Log$_e$ growth delay, days	Growth rate, cm^3/day	Log$_e$ survival time, days
0	0.87 ± 0.15	0.29 ± 0.04	4.12 ± 0.18
5	2.08 ± 0.10	0.10 ± 0.01	4.30 ± 0.18
15	3.14 ± 0.13	0.10 ± 0.01	4.41 ± 0.16
25	3.89 ± 0.19	0.05 ± 0.00	4.89 ± 0.14

[a] Data are expressed as mean ± SEM.

TABLE 3.—Tumor growth following treatment by doxorubicin only[a]

Dose, mg/kg	Log$_e$ growth delay, days	Growth rate, cm^3/day	Log$_e$ survival time, days
0	1.42 ± 0.07	0.15 ± 0.02	4.73 ± 0.24
2	1.40 ± 0.15	0.16 ± 0.02	4.23 ± 0.21
4	1.77 ± 0.10	0.10 ± 0.02	4.42 ± 0.11
9	1.80 ± 0.09	0.10 ± 0.02	4.06 ± 0.16

[a] Data are expressed as mean ± SEM.

This design represents a total of 90 experimental cells, i.e., distinct treatment combinations ($3 \times 3 \times 2 \times 5 = 90$). With a replication factor of 5, the experimental design requires a total of 450 experimental tumor-bearing animals, each receiving 1 doxorubicin and 1 radiation treatment.

Only a single treatment with each agent was studied because of the additional complexity in experimental design that would be necessitated by inclusion of multiple treatments with each agent. We considered such multiple treatments excessively complex and, as such, the subject of potential future investigation based upon a considerably restricted combination of the factors tested in the present study.

Because it is logistically difficult for investigators to conduct the complete experimental design as depicted in table 1 at one time, the overall design was segmented by replicate. One segment of the design consisted of 90 tumor-bearing animals, each representing one of the treatment combinations (i.e., experimental cells). In other words, one segment, which was done at one time, was one complete replicate of the overall design. In addition, 5 untreated tumor-bearing rats were included with each segment as a segment control, outside the statistical design, but which could be used for assurance that the tumor growth was following the expected time profile.

Analysis of tumor growth data.—Tumor growth was analyzed on an individual animal basis by the plotting of the tumor volume as a function of time after initiation of treatment. The growth of each tumor was fit by the method of least squares to a quadratic exponential growth model:

$$\log (V_t) = a + bt + ct^2,$$

where V_t = tumor volume at time t, t = time, and a, b, and c = regression constants. From this fit, two values were calculated, the growth delay (defined to be the number of days required for the tumor to reach twice its initial volume) and the growth rate (defined as the slope of the growth curve at the time of first doubling). The growth delay represents a measurement of the length of time that a therapy provides control over the growth of the tumor. The growth rate, at the point when a therapy no longer provides tumor control (the growth delay time), represents a measurement of the slope of the growth curve at a "fixed" tumor volume.

RESULTS

Single Agent Dose Versus Response Studies

The effects of doxorubicin at 2, 4, or 9 mg/kg or radiation at 5, 15, or 25 Gy on the growth of the Dunning R3327G tumor are presented in figures 1 and 2 and tables 2 and 3. These 3 doxorubicin doses were selected from a more exhaustive series of doses (11 doses; 0.5 to 30 mg/kg), based on the criteria outlined in the Materials and Methods section. The low, medium, and high doses were 2 4, and 9 mg/kg, respectively.

These 3 radiation doses were selected from a larger dose series (12 doses; 5 to 60 Gy), as already described. At 5 Gy, there was a modest delay in tumor growth, which increased further at 15 and 25 Gy. At doses larger than 25 Gy, tumor control was even better, resulting in occasional cures at doses over 40 Gy. Side effects, notably epilation and dry and moist desquamation, were observed, beginning at 20

TABLE 4.—Analysis of tumor growth following doxorubicin and radiation combined

	Probability[a]			
	Growth delay		Growth rate	
	Doxorubicin		Doxorubicin	
Source of variability[b]	2 levels[c]	3 levels	2 levels[c]	3 levels
Doxorubicin	.0001	.0001	.0179	.0001
Radiation	<.0001	<.0001	.0001	.0012
Delay	.0001	.0001		
Sequence	.0128	.0112		
Doxorubicin and radiation		.0001		.0001
Sequence and delay	.0002	.0001		
Sequence and radiation	.0252			
Sequence, delay, and doxorubicin			.0204	

[a] Source of variability is significant. Missing values have $P > .05$.

[b] All sources of variability were tested (single factor, and two-, three-, and four-way interactions). Only those yielding $P < .05$ are listed.

[c] All animals receiving high-dose doxorubicin were excluded from analysis.

TABLE 5.—Summary of individual treatment means for growth delay[a]

		Sequence					
		Doxorubicin + radiation			Radiation + doxorubicin		
		Radiation, Gy[c]			Radiation, Gy[c]		
Doxorubicin, mg/kg	Delay, hr[b]	5	15	25	5	15	25
2	0.5	1.78	2.84	2.62	1.78	2.81	3.63
	12	1.76	2.94	3.70 (4)	0.90	2.59	3.71 (4)
	24	1.79	2.86	3.82	1.68 (4)	3.10	3.09
	48	1.67	2.01	3.06	1.36	2.42	3.24
	120	0.99	1.65	1.44	1.78	2.60	3.29
4	0.5	1.62	2.89	3.39	1.89	3.26	3.22 (4)
	12	1.55	3.59	3.55 (4)	2.09	3.07	3.75
	24	1.85	2.92	3.31 (4)	1.92	2.66	3.39
	48	2.35	2.97	3.33	1.87	3.15	3.97
	120	1.73	2.41	2.32	1.07	3.02	3.72
9	0.5	1.95 (3)	1.95 (4)	2.69 (3)	2.22	2.12 (3)	2.87 (4)
	12	2.40 (4)	2.59 (2)	3.12 (2)	2.09 (4)	2.58 (3)	2.34 (2)
	24	2.15 (3)	2.18 (3)	2.54 (4)	1.78	2.41 (2)	2.51 (4)
	48	1.78 (4)	2.32 (3)	2.44 (2)	2.13 (4)	2.53 (3)	2.50 (3)
	120	2.01	1.82 (4)	1.94 (2)	2.00	2.62 (3)	2.55 (3)

[a] *Numbers in parentheses* are the numbers of animals comprising the mean (5 unless otherwise specified).
[b] Delay is the time between administration of the first and second agents.
[c] Values are expressed as the $\log_e$ of growth delay in days.

Gy and increasing in severity with dose. The high dose was limited to 25 Gy because 1) significant tumor control was evident, 2) skin reactions were becoming apparent, and 3) some room for enhancement of tumor control by doxorubicin synergism was available. At doses larger than 25 Gy, it was believed that tumor control by radiation alone was so pronounced that demonstration of drug enhancement would be difficult.

Interaction of Doxorubicin and Radiation

We performed 5 experiments to study the interaction of doxorubicin and radiation in the Dunning R3327G rat prostatic adenocarcinoma. Each experiment represents 1 replicate of each of the 90 distinct treatment combinations suggested in table 1, making a total of 5 replicates of each treatment. The growth delay (time required for the tumor

TABLE 6.—Summary of individual treatment means for growth rate[a]

		Sequence					
		Doxorubicin + radiation			Radiation + doxorubicin		
		Radiation, Gy[c]			Radiation, Gy[c]		
Doxorubicin, mg/kg	Delay, hr[b]	5	15	25	5	15	25
2	0.5	0.127	0.047	0.051	0.113	0.065	0.028
	12	0.093	0.059	0.045 (4)	0.300	0.061	0.054 (4)
	24	0.100	0.044	0.040	0.106 (4)	0.067	0.049
	48	0.119	0.076	0.043	0.135	0.059	0.048
	120	0.119	0.142	0.095	0.071	0.068	0.039
4	0.5	0.148	0.057	0.049	0.113	0.052	0.036 (4)
	12	0.112	0.057	0.050 (4)	0.086	0.053	0.041
	24	0.095	0.056	0.049 (4)	0.093	0.062	0.043
	48	0.084	0.039	0.030	0.072	0.040	0.027
	120	0.069	0.048	0.059	0.153	0.047	0.033
9	0.5	0.104 (3)	0.129 (4)	0.233 (3)	0.072	0.060 (3)	0.125 (4)
	12	0.126 (4)	0.127 (2)	0.156 (2)	0.097 (4)	0.152 (3)	0.080 (2)
	24	0.123 (3)	0.247 (3)	0.093 (4)	0.085	0.226 (2)	0.176 (4)
	48	0.167 (4)	0.085 (3)	0.238 (2)	0.091 (4)	0.169 (3)	0.180 (3)
	120	0.089	0.089 (4)	0.102 (2)	0.081	0.083 (3)	0.121 (3)

[a] *Numbers in parentheses* are the numbers of animals comprising the mean (5 unless otherwise specified).
[b] Delay is the time between administration of the first and second agents.
[c] Values are expressed as growth rate in cubic centimeters/day.

TABLE 7.—Means for interaction of doxorubicin and radiation

Doxorubicin, mg/kg	Radiation, Gy	Log growth delay, $\log_e$ in days	Growth rate, cm^3/day
2	5	1.55	0.129
	15	2.58	0.069
	25	3.14	0.049
4	5	1.79	0.103
	15	2.99	0.051
	25	3.40	0.042
9	5	2.05	0.096
	15	2.27	0.132
	25	2.57	0.149

TABLE 8.—Means for interaction of sequence and delay

Delay, hr	Sequence	Log growth delay, $\log_e$ in days
0.5	Doxorubicin + radiation	2.44
	Radiation + doxorubicin	2.65
12	Doxorubicin + radiation	2.75
	Radiation + doxorubicin	2.57
24	Doxorubicin + radiation	2.63
	Radiation + doxorubicin	2.53
48	Doxorubicin + radiation	2.46
	Radiation + doxorubicin	2.59
120	Doxorubicin + radiation	1.80
	Radiation + doxorubicin	2.51

to reach twice its size at the time of treatment) was calculated on an individual animal basis by our fitting a quadratic exponential equation to the relevant measurements of time and tumor volume. The growth rate of the tumor, at the time of first doubling, was also calculated from the fitted curve.

Analysis of variance was performed on both the untransformed and $\log_e$ transformed growth delay times. The factors and their interactions found statistically significant ($P<.05$) were the same for both forms of the data, although the log transformation provided a better fit to the data and therefore is reported here. The results of the statistical analysis are summarized in table 4, which lists those sources of variability found significant. Tables 5 and 6 present the individual treatment means for growth delay and growth rate, respectively. Animal mortality was not of consequence, except for the rats given the high doxorubicin dose of 9 mg/kg; a considerable number of them died from drug toxicity (*see* table 5). Because of possible biasing in the analysis of variance associated with this toxicity, the analysis was run both with and without the high-dose doxorubicin data. As shown in table 4, this did not alter the importance of the individual factors of doxorubicin, radiation, treatment delay, or sequence, or the interaction of sequence and delay in the treatment effect on growth delay. In contrast, a highly significant ($P=.0001$) interaction between doxorubicin and radiation was evident when 3 levels of drug were considered, but not when only 2 levels were tested.[5]

The nature of the interaction of doxorubicin and radiation can be seen from the table of means (table 7). At each drug dose, the growth delay increases with increasing radiation dose. In contrast, at each radiation dose the growth delay increases with drug dose for the low radiation dose, whereas at the medium and high radiation doses, the growth delay peaks with the medium dose of drug. The high drug dose shows a decline in the growth delay. This decline in growth delay with high drug dose is likely the result of a bias in the data associated with drug mortality. Those animals whose tumors were growing rapidly would have their tumors double before death, but those whose tumors were growing more slowly would die before the tumors doubled. Because tumors that have no doubling time are censured (deleted) by the analysis, this has the effect of producing a doubling time (growth delay) mean smaller than it should be. That this actually happened is clear from examination of the growth rate means in table 7; the growth rate decreases with increasing radiation dose (as it should) for the low and medium drug doses but increases with radiation dose for the high drug dose.

The interaction of sequence and delay is explored in table 8. For the sequence of radiation followed by doxorubicin, the means remain approximately the same, whereas the reverse sequence, doxorubicin followed by radiation, exhibits a drop in mean growth delay with a treatment delay of 120 hours. This interaction reflects the poor control in tumor growth produced by doxorubicin, and that, with a doubling time of 2–3 days in untreated tumors, the tumors will grow considerably during the 5-day period between drug and radiation treatments.

In the case of the sequence and radiation interaction, the table of means (table 9) shows that growth delay increases more rapidly with dose for radiation first than when doxorubicin was given first.

The only third-order interaction was sequence, delay, and doxorubicin (table 10). For doxorubicin preceding radiation, there is an increase in growth rate with drug dose up to 24 hours delay, after which the growth rate was

TABLE 9.—Means for interaction of sequence and radiation

Radiation, Gy	Sequence	Log growth delay, $\log_e$ in days
5	Doxorubicin + radiation	1.71
	Radiation + doxorubicin	1.63
15	Doxorubicin + radiation	2.71
	Radiation + doxorubicin	2.86
25	Doxorubicin + radiation	3.02
	Radiation + doxorubicin	3.50

---

[5] Note of explanation to nonstatistically minded readers: The means presented in tables 7 to 10 are based on the same data as the individual treatment means of tables 5 and 6. The individual animal observations have been averaged only for replicates (i.e., animals receiving identical treatments) in tables 5 (growth delay) and 6 (growth rate); each value is the average of the responses of 2 to 5 animals. In the case of a significant statistical interaction, one can see the nature of that interaction (i.e., how the response changes as the interacting factors change) more clearly by calculating means irrespective of all factors *except* those interacting. Tables 7 to 10 present means recalculated for each significant interaction.

TABLE 10.—Means for interaction of sequence, delay, and doxorubicin

Delay, hr	Sequence	Doxorubicin, mg/kg[a]	
		2	4
0.5	Doxorubicin + radiation	0.075	0.084
	Radiation + doxorubicin	0.069	0.069
12	Doxorubicin + radiation	0.067	0.075
	Radiation + doxorubicin	0.144	0.060
24	Doxorubicin + radiation	0.062	0.068
	Radiation + doxorubicin	0.071	0.066
48	Doxorubicin + radiation	0.079	0.051
	Radiation + doxorubicin	0.080	0.046
120	Doxorubicin + radiation	0.119	0.059
	Radiation + doxorubicin	0.059	0.078

[a] Values are expressed as cubic centimeters/day.

observed to decline with increasing drug dose. For the reverse sequence, radiation first, there is no clear trend associated with delay or drug dose.

DISCUSSION

The interaction of doxorubicin and radiation has been systematically investigated in the Dunning R3327G prostatic adenocarcinoma, the preeminent model of the human disease. The end points examined are growth delay (the time required for tumor volume to increase to twice the volume at treatment) and growth rate (the slope of the volume growth curve at the time of first doubling). Growth delay represents a clinically relevant measure of the length of time that a particular therapy provides "control" of tumor growth, i.e., prevents the tumor from exceeding twice its initial volume. We have defined it as time to double the volume at treatment, rather than time to reach a specified volume, in an attempt to reduce the biasing of calculated delay times due to variation in initial tumor volume among animals. In contrast, growth rate, measured at the time the tumor achieved twice its starting volume, represents the slope of the volume growth curve at a similar volume for all tumors. It measures the effect of a therapy on the rate of increase in tumor size at a "fixed" volume.

Used individually, both doxorubicin and radiation provided dose-dependent delay in tumor growth (fig. 1 and 2, tables 2 and 3); radiation was considerably more efficacious under the conditions tested. Survival time was found to be an unsatisfactory measure of treatment effectiveness. Following radiation, survival time increased in a dose-dependent manner; on the contrary, after doxorubicin, survival time decreased in a manner not related to drug dose. Large R3327G tumors grown subcutaneously necrose through the skin, producing ulcerating lesions; necropsies suggest that animals die from infection and hemorrhage rather than tumor burden.

When radiation and doxorubicin are combined in a factorial experimental design (table 1) including the factors, agent dose, sequence, and delay between agents, statistical analysis permits partitioning the overall variability to identify that portion attributable to each factor, as well as the various interactions of factors.

Analysis of variance of growth delay (period of tumor control by therapy) showed that each factor (doxorubicin, radiation, sequence of agents, and delay between agents) was highly significant, as were certain second-order interactions of these factors (i.e., doxorubicin and radiation, sequence and delay, and sequence and radiation). No third- or fourth-order interactions were significant (*see* table 4). The doxorubicin and radiation interaction is explained by the biasing of the growth delay due to doxorubicin toxicity; the mean growth delay after high-dose drug is deceptively short, due to death before an animal's tumor had doubled (table 7). The interaction of sequence and delay is interpreted as growth of the tumor between treatments when doxorubicin was given first (table 8). Even the low dose of radiation gave longer control of this tumor than did the high dose of doxorubicin, 8.0 days (table 2, $e^{2.08}$) versus 6.0 days (table 3, $e^{1.80}$); following doxorubicin, the tumor would have almost doubled by 5 days (120 hr) before the radiation treatment was given. In fact, occasional tumors treated with drug first did double before the radiation was administered at 5 days. The sequence and radiation interaction is interesting because it suggests that at higher radiation doses, radiation is better potentiated by doxorubicin if the radiation is given first, but, at lower radiation doses, sequence makes little difference (table 9).

Analysis of variance of the tumor growth rates identified fewer significant effects (table 4); only the primary factors of doxorubicin and radiation were significant. One second-order interaction (doxorubicin and radiation) and one third-order effect (sequence, delay, and doxorubicin) were detected. The doxorubicin and radiation effect has been attributed to animal mortality (drug induced) before tumor doubling with the result of censure of the data from the affected animals. The third-order interaction, sequence, doxorubicin, and delay (table 10) showed that growth rate increased with drug dose up to 24 hours delay and then was observed to decline with increased drug dose when doxorubicin preceded radiation, but no clear trend existed for the reverse sequence.

The method of data reduction when growth delay was used has eliminated the initial tumor volume at the time of treatment from appearing directly in the analysis. To further reduce any potential influence of differing initial volumes on the analysis, we treated it as a covariate, which resulted in an adjusted analysis with variability related to starting volume differences being further reduced by this approach.

Analysis of the segment controls for the 5 replicate studies required to produce the full design presented in table 1 identified only minor fluctuations not considered to be of significance. This procedure is believed to validate satisfactorily the concept of proper segmentation of a large study such as the one presented here to reduce a large experimental design to pieces (segments) of size suitable for execution.

Tumor immunogenicity is clearly not a factor in this tumor model, as it has been shown to be nonimmunogenic (*16*).

It is tempting for one to compare the doubling times achieved with radiation alone (table 2) with the growth delays obtained with the 90 different treatments (table 5), identifying those treatments yielding growth delays enhanced by doxorubicin. For example, 15 Gy of radiation alone results in a growth delay of 23 days ($e^{3.14}$); 4 mg doxorubicin/kg followed after 12 hours by 15 Gy of radiation gives a growth delay of 36 days ($e^{3.59}$), an apparent 50% increase in tumor control. Great caution must be used because the analysis of variance for 2 levels of doxorubicin yielded $P = .5037$ for the interaction of doxorubicin and radiation. The analysis argues that the effects on this tumor of doxorubicin and radiation are basically additive, with neither negative (antagonistic) or positive (synergistic) interactions. Whereas this finding agrees with the majority of other studies [reviewed in (*14*)], our study is one of interaction at the clinical level; interpretation of biochemical mechanisms should be done with great caution. Although synergism between doxorubicin and radiation was not observed, the present results demonstrate that the factors of sequence and time delay between agents are important considerations in the observed clinical effect on this tumor.

REFERENCES

(*1*) Block NL, Camuzzi F, Denefrio J, et al: Chemotherapy of the transplantable adenocarcinoma (R-3327) of the Copenhagen rat. Oncology 34:110–113, 1977.

(*2*) Block NL, Camuzzi F, Stover B, et al: Further experience with chemotherapy in the Dunning prostatic adenocarcinoma. Trans Am Assoc Genitourinary Surg 70:57–59, 1979.

(*3*) Camuzzi F, Gottlieb C, Block NL, et al: Investigation of different combinations of estrogen therapy and radiation therapy on the prostatic adenocarcinoma (R-3327). Urology 15:443–447, 1980.

(*4*) Claflin AJ, McKinney EC, Fletcher MA: The Dunning R-3327 prostate adenocarcinoma in the Fischer-Copenhagen F_1 rat. A useful model for immunological studies. Oncology 34:105–109, 1977.

(*5*) Pollack A, Block NL, Stover BJ, et al: Tumor progression in serial passages of the Dunning R3327-G rat prostatic adenocarcinoma: Growth rate response to endocrine manipulation. Cancer Res 45:1052–1057, 1985.

(*6*) Shessel FS, Block NL, Stover B, et al: Endocrine manipulation in the Dunning prostatic adenocarcinoma. Invest Urol 17:529–533, 1980.

(*7*) Smolev JK, Heston WD, Scott WW, et al: Characterization of the Dunning R-3327-H prostatic adenocarcinoma: An appropriate animal model for prostatic cancer. Cancer Treat Rep 61:273–287, 1977.

(*8*) Weissman RM, Coffey DS, Scott WW: Cell kinetic studies of prostatic cancer: Adjuvant therapy in animal models. Oncology 34:133–137, 1977.

(*9*) Handelsman H: The limitations of model systems in prostatic cancer. Oncology 34:96–99, 1977.

(*10*) Higuchi M: On the significance of sex hormones in producing experimental prostate tumor in the rat. Recent Results Cancer Res 60:27–52, 1977.

(*11*) Council on Scientific Affairs: Combined modality approaches to cancer therapy. JAMA 251:2398–2407, 1984.

(*12*) DeVita VT, Lippman M, Hubbard SM, et al: The effect of combined modality therapy on local control and survival. Int J Radiat Oncol Biol Phys 12:487–501, 1986.

(*13*) Krakoff IH: Cancer chemotherapeutic agents. CA 27: 130–143, 1977.

(*14*) Bellamy AS, Hill BT: Interactions between clinically effective antitumor drugs and radiation in experimental systems. Biochim Biophys Acta 738:125–166, 1984.

(*15*) Janik P, Briand P, Hartman NR: The effect of estrone-progesterone treatment on cell proliferation kinetics of hormone-dependent GR mouse mammary tumors. Cancer Res 35:3698–3704, 1975.

(*16*) Claflin AJ, McKinney ED, Fletcher MA: Immunological studies of prostate adenocarcinoma in an animal model. *In* Models for Prostate Cancer. New York: Alan R Liss, 1980, pp 365–377.

Discussion Summary

Ian F. Tannock[1,*] **and William F. Sindelar**[2]

ALTERNATING TREATMENT WITH CHEMOTHERAPY AND RADIATION

Several comments relating to Dr. Looney's presentation suggested than an improved therapeutic index could be obtained from the use of radiation and chemotherapy in combination. Although some of the clinical data for Hodgkin's disease and small cell lung cancer suggest good results from the use of alternating therapies, it was pointed out that only through a randomized prospective clinical trial that compared alternating therapy with either single modality or sequential therapies could a definitive answer be obtained relating to therapeutic benefit. The possible mechanisms that might lead to therapeutic benefit through the use of alternating therapy were also discussed. Dr. Looney had introduced the Goldie–Coldman hypothesis whereby alternating therapy could minimize the emergence of cells resistant to either modality. However, this requires that there is no cross-resistance between treatment with radiation and with the selected drugs, and also that mutation is the major mechanism leading to therapeutic resistance; evidence on both of these points is lacking. Other potential mechanisms include the inhibition of repopulation by chemotherapy that might otherwise occur between fractions of radiation.

TREATMENT DIRECTED AT TUMOR SUBPOPULATIONS

A recurring theme in the discussion was that the models used to study drug–radiation interactions had been rather crude, and only recently have methods emerged that allowed the study of subpopulations that arise as a result of several types of tumor heterogeneity. Important subpopulations were those of aerobic or hypoxic cells, cells at low or normal pH, and cells in different proliferative states. Heterogeneity usually sets the tumor at a disadvantage with respect to more uniform normal tissues, but therapeutic advances will probably require the use of therapies directed at specific subpopulations in an attempt to overcome this disadvantage. Various fractionation methods have been described in the posters, including the use of flow cytometry in conjunction with vital staining and the use of centrifugal elutriation. However, Dr. Durand pointed out the limitations of the models and methodology. For example, in his studies of spheroids, the effects that he could measure against individual subpopulations were not always predictive of the response of the whole spheroid. There was further discussion of appropriate models, and the question was raised as to whether rapidly growing tumors in animals might adequately model the distribution of oxygen or pH that would occur in human tumors. Others suggested that the limitation lay not in the models but in our previous inability to use them correctly. Certainly, the participants agreed that, in future studies, investigators should try to analyze therapeutic results against constituent subpopulations to derive a more mechanistic approach to treatment of the whole tumor.

PHARMACOKINETICS

Several points were raised about the use of intratumoral injections of anticancer drugs and especially the use of drugs with a matrix that prevents their rapid dispersal. Dr. Siemann suggested that a therapeutic gain had been demonstrated for intratumoral as opposed to systemic injection, but the use of the matrix did not appear to add further therapeutic gain, at least in the results reported by Dr. Begg and colleagues. Dr. Begg agreed with this when looking at drug treatment alone, but he stated that additional data were available that suggested that the matrix formulation would be useful in radiation–drug interactions because delayed clearance of the drug would then make these much less dependent on the schedule used. Others commented on the use of radiation to change the properties of the blood vessels that might then allow better entry of drugs into the tumor. Examples of this include clinical evidence for increased levels of methotrexate in the central nervous system when this drug is given after radiation therapy in the treatment of acute lymphocytic leukemia. This, however, may be a variable effect; others have found a decrease in blood supply after radiation, and when it does occur, it may not give a therapeutic advantage because the sequence of radiation and methotrexate has probably also led to increased toxicity in the form of leukoencephalopathy. Some participants commented on Dr. Douple's results that treatment with radiation could lead to increased concentration of carboplatin in tumors. This effect appears to be specific for carboplatin and does not occur for cisplatin; its mechanism is uncertain. It was stressed that a comparison of normal tissue levels will be important for concluding that this effect is therapeutically useful. Dr. Fu and others cautioned that the influence of radiation on drug levels would probably be dependent on radiation dose, dose rate, and the type of drug because results were variant in basic and clinical literature.

---

[1] Department of Medicine, Ontario Cancer Institute, Toronto, Canada.

[2] Surgery Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[*] *Reprint requests:* Ian F. Tannock, M.D., Department of Medicine, Ontario Cancer Institute, 500 Sherbourne St., Toronto, Ontario M4X 1K9, Canada.

Dr. Twentyman continued the discussion of vascular effects and asked for opinions about the possibility of selective killing of endothelial cells in tumors, a strategy that has been pursued by Dr. Denekamp and others. Another researcher pointed out that regrowth of tumors after radiation was usually slower than the growth of untreated tumors and this was thought to be due to vascular damage. In contrast, regrowth of tumors after drug treatment tended to be similar to that in the untreated tumor, which suggests that vascular proliferation was generally less inhibited by drugs. However, the fact that some drugs, such as doxorubicin, have limited penetration from blood vessels might still make vascular damage a distinct possibility in chemotherapy. The antivascular therapy described by Folkman, who used heparin fragments and glucocorticoids, was also mentioned, although there appears to be poor reproducibility between systems.

In general, participants in the discussion reemphasized points made in introductory talks that we had to advance from a study of phenomena to a study of mechanisms; they stressed the importance of tumor subpopulations in analyses of the interactions between radiation and drugs.

Mechanisms of Interaction/Resistance—II

Keynote Address: Mechanisms of Cross-resistance Between Radiation and Antineoplastic Drugs

Robert F. Ozols,* Hidetaka Masuda, and Thomas C. Hamilton[1]

ABSTRACT—The mechanisms responsible for the cross-resistance between radiation and certain antineoplastic agents have been examined in human ovarian cancer cell lines. Cell lines established from patients at a time when they were resistant to combination chemotherapy regimens, which included cisplatin and an alkylating agent as well as cell lines with resistance induced in vitro to melphalan and cisplatin, all have increased cellular levels of glutathione (GSH) compared with drug-sensitive cell lines from untreated patients. In addition, cell lines with acquired resistance to melphalan and cisplatin, but not to doxorubicin, were cross-resistant to radiation. L-Buthionine sulfoximine (BSO), an irreversible inhibitor of γ-glutamylcysteine synthetase, lowered GSH levels in all the resistant cell lines studied. Lowering of GSH levels to less than 10% of control values potentiated the in vitro cytotoxicity of melphalan and cisplatin. Furthermore, BSO was also shown to potentiate the cytotoxicity of melphalan in a nude mouse model system of ovarian cancer in which mice die of disseminated intra-abdominal carcinomatosis. The BSO administered in the drinking water decreased GSH levels by 96%. A single melphalan treatment of 5 mg/kg following GSH depletion produced a 72% increase in median survival time compared with treatment with melphalan alone. In addition, depletion of GSH levels in cell lines with acquired resistance to melphalan led to a marked sensitization of these cells to irradiation. The dose increment required to reduce the surviving fraction by a factor of 1/e on the exponential portion of the dose-response curve decreased from 143 to 95. In cell lines with induced resistance to melphalan and cisplatin, there was a marked increase in DNA repair compared with the parent sensitive cell line. These results indicate that a common repair mechanism may be responsible for the primary resistance to alkylating agents, cisplatin, and irradiation. They also demonstrate that lowering GSH levels with BSO may be of potential clinical benefit in the treatment of drug-resistant and radiation-resistant ovarian cancer patients.—NCI Monogr 6: 159-165, 1988.

The development of resistance to chemotherapeutic agents has limited the overall effectiveness of this modality of treatment in a variety of solid tumors. In some tumors, development of drug resistance has been correlated with clinical resistance to radiation. Ovarian cancer represents perhaps the clearest clinical situation of this phenomenon involving cross-resistance between antineoplastic drugs and radiation. It has been shown that radiation therapy is effective for patients with small volume residual disease left after the initial laparotomy (*1*). However, when similar techniques of radiation therapy are used after the patients have been treated with combination chemotherapy and are left with small volume residual disease, Hoskins et al. (*2*) and Malcolm and associates (*3*) have demonstrated that total abdominal radiation therapy is not capable of inducing a significant number of complete responses and a prolongation of survival. The mechanisms responsible for the development of cross-resistance between radiation and some antineoplastic drugs remain to be established. Recent studies on the molecular pharmacology of antineoplastic drugs in human tumor cell lines and other experimental systems of cancer have revealed that there are significant parallels between the cytotoxicity of irradiation and alkylating agents and that tumor cells may acquire mechanisms of resistance capable of decreasing the cytotoxic effects of some antineoplastic drugs as well as of radiation.

In this paper, we shall first review the mechanisms responsible for the development of broad cross-resistance (pleiotropic drug resistance) that have been identified in some experimental model systems of cancer. We shall then focus on the roles of GSH and DNA repair as mechanisms responsible for the cross-resistance that may exist in certain clinical situations among alkylating agents, cisplatin, and radiation.

PLEIOTROPIC DRUG RESISTANCE

Tumor cells can become resistant to the cytotoxic effects of chemotherapy in many ways, and these have been reviewed in detail in recent publications (*4,5*). Table 1 contains a summary of the major different mechanisms of resistance that have been described.

Some cell lines that have been selected for resistance to a single agent, particularly a vinca alkaloid or an anthracycline, develop cross-resistance to a wide variety of structurally dissimilar drugs. This type of broad cross-resistance has been termed "pleiotropic" (multidrug) resistance. The mechanism(s) whereby human tumor cells develop this phenotype of drug resistance remain(s) to be completely elucidated.

The mechanism of multidrug resistance in CHO cells has been extensively studied by several investigators (*6-9*). The development of resistance to a drug, such as colchicine, in

---

ABBREVIATIONS: GSH = glutathione; CHO = Chinese hamster ovary; P170 = 170,000-dalton glycoprotein; BSO = L-buthionine sulfoximine; IC_{50} = concentration which caused 50% inhibition of growth; LD_{10} = dose lethal to 10% of animals; D_0 = dose increment required to reduce the surviving fraction by a factor of 1/e on the exponential portion of the dose-response curve; $\bar{n}$ = exponential number; UDS = unscheduled DNA synthesis.

[1] Experimental Therapeutics Section, Medicine Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

* *Reprint requests:* Robert F. Ozols, M.D., Experimental Therapeutics Section, Medicine Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bldg. 10, Rm. 12N226, National Institutes of Health, Bethesda, MD 20892.

Table 1.—Mechanisms of drug resistance

Mechanism	Drug
Alterations in:	
Drug uptake (carrier mediated)	Methotrexate Melphalan Mechlorethamine
Intracellular drug accumulation	Doxorubicin Vinca alkaloids Dactinomycin
Cellular metabolism	Cytarabine 5-Fluorouracil Methotrexate 6-Mercaptopurine
Cofactor levels	6-Thioguanine Floxuridine
Cellular repair	Alkylators Nitrosoureas
Decreased drug activation	Cytarabine 5-Fluorouracil 6-Mercaptopurine 6-Thioguanine Methotrexate
Increased metabolic inactivation	6-Mercaptopurine 6-Thioguanine Cytarabine Alkylators Cisplatin
Increased levels of target proteins	Methotrexate Phosphonacetyl-L-aspartate Floxuridine Pentostatin

this experimental system leads to a decrease in cellular accumulation of other drugs, such as doxorubicin and vincristine. Furthermore, it has been demonstrated that the drug-resistant cells express a membrane glycoprotein (P-glycoprotein), which is nondetectable in drug-sensitive cells. The levels of P170 correlate with the overall degree of drug resistance in these cells. Thus it has been postulated that the drug-resistant phenotype is due to the expression of a membrane protein that is responsible for a decrease in net accumulation of other drugs and a corresponding decrease in cytotoxicity.

The genetic nature of this alteration has recently been studied extensively. Debenham et al. (*7*), using calcium phosphate-mediated DNA transfection, showed that DNA from resistant CHO cells is able to transfer resistance to sensitive cells. They further demonstrated that the transfected cells express increased levels of P170. The observation that levels of specific proteins such as P170 were increased in some pleiotropic drug-resistant cells suggests that the amplification of genes coding for these proteins is responsible for those changes (*8*). It has been frequently reported that cytogenic abnormalities were consistent with gene amplification sequences present in the cells exhibiting pleiotropic drug resistance. Thus both double minutes and homogeneous staining regions have been described in pleiotropic drug-resistant cells, and in some cases the number of double minutes correlates with the level of drug resistance.

Recent experimental evidence has further suggested a role for gene amplification in the development of pleiotropic drug resistance. Roninson (*9*), using a special technique to detect amplified DNA sequences that involves repetitive rounds of DNA–DNA hybridization in agarose, detected amplified DNA sequences in pleiotropic-resistant CHO cells that are not present in the drug-sensitive cells. Similarly, Riordan and co-workers (*10*), using a cDNA clone encoding part of P170, demonstrated that gene amplification occurred in CHO drug-resistant cells. Furthermore, Bliek et al. (*11*), at The Netherlands Cancer Institute, also demonstrated the overexpression and amplification of 5 genes in a multidrug-resistant CHO cell line.

The relevance of these findings to human tumor cells in the development of pleiotropic drug resistance in clinical situations is less clear. When Bell and his associates (*12*) used a monoclonal antibody directed against the P170 purified from drug-resistant CHO cells and from drug-resistant human CEM cells, they detected a high-molecular-weight membrane protein in 2 of 5 patients with ovarian cancer who were undergoing therapy. Although this was an important observation correlating resistance with membrane protein changes, the frequency with which any change in membrane proteins occurs in clinical drug resistance remains to be established. It is apparent that if genes associated with the development of the multidrug resistance phenotype can be identified, they will provide researchers a molecular probe with which to screen tumors for biochemical evidence of drug resistance. Furthermore, this would also raise the possibility of specific biochemical, immunologic, and genetic approaches to prevent or reverse clinical drug resistance.

However, it must be emphasized that the importance of this major mechanism of multidrug resistance in the Chinese hamster cell lines (i.e., a decreased net accumulation of structurally dissimilar drugs) remains to be determined in human tumor cells. We have previously demonstrated that a human ovarian cancer cell line made resistant in vitro to doxorubicin developed a pattern of cross-resistance similar to the CHO cells and also had markedly less accumulation of drug compared with the sensitive cell line from which it was derived (*13*). However, human ovarian cancer cell lines established from patients treated with combination chemotherapy regimens (which included doxorubicin) have no significant alterations in accumulation of this drug (*14*). This finding suggests that alternative mechanisms of drug resistance are playing a role in these cells other than a decreased net accumulation of anthracyclines. Furthermore, the pleiotropic drug resistance in CHO cells primarily relates to broad cross-resistance between natural products. In contrast, broader patterns of cross-resistance frequently are present in clinical situations. For example, in patients with ovarian cancer who are resistant to non-doxorubicin-containing combination chemotherapy regimens or to melphalan, second-line therapy with doxorubicin is ineffective (*15*). Thus the mechanisms responsible for clinical broad cross-resistance appear to be multifactorial and may involve other mechanisms in addition to a decreased net accumulation of drugs.

ROLE OF GLUTATHIONE IN DRUG RESISTANCE AND RESISTANCE TO RADIATION

That an association exists between radiation resistance and elevations in cellular GSH levels is known. There are at least two major pathways by which GSH levels may affect radiosensitivity (*16*): 1) detoxification of radiation-induced toxins including free radicals and 2) involvement in the repair of critically damaged DNA sites. It has been shown that GSH can potentially eliminate harmful free radicals by hydrogen atom donation via one-electron reactions. Evidence also shows that GSH is involved in facilitating the repair of radiation. However, the precise nature of the molecular mechanisms wherein GSH modulates radiation cytotoxicity remains to be established.

Because GSH is involved in a variety of critical cellular functions including metabolism, transport, and protection from toxic compounds, its role in the protection from antineoplastic drugs has also been investigated. Relle-Somfai et al. (*17*) demonstrated that L1210 cells resistant to melphalan had a twofold to fourfold higher level of GSH than the sensitive cell lines from which they were derived. Previously, it had been reported that other cell lines including nitrogen mustard-resistant Yoshida sarcoma cells also had elevated levels of GSH (*18*). These observations were of particular potential clinical relevance because pharmacologic ways exist in which GSH metabolism can be manipulated. Various agents have been used to decrease cellular GSH levels, and until recently the lack of specificity as well as the toxicity had not made these agents suitable candidates for clinical manipulation of GSH levels. For example, reduction in GSH can be accomplished readily with the thiol reactive agent diethyl maleate, and the reaction occurs either nonenzymatically or through the action of GSH S-transferases. However, the usefulness of this approach is limited by the nonspecific effects of diethyl maleate on cytoconstituents and metabolic processes. In addition, rapid depletion of GSH is likely to be followed by rapid resynthesis to pretreatment levels (*19*). In contrast, the inhibition of GSH synthesis by the synthetic amino acid BSO is of potential clinical relevance because BSO irreversibly inhibits the enzyme γ-glutamylcysteine synthetase and leads to a marked reduction in cellular GSH levels. Initial studies by Griffith and Meister (*20*) demonstrated that mice can tolerate the administration of BSO for extended periods, even though it does lead to a transient depletion of GSH in the kidney, liver, and plasma. Relle-Somfai and co-workers (*17*) determined that BSO reduced GSH levels in melphalan-resistant L1210 cells and restored sensitivity to melphalan. Resistant cells were shown to dechlorinate melphalan to a less toxic intermediate (*21*), which suggests that a possible mechanism for resistance to melphalan in L1210 cells was the metabolism of melphalan to a less cytotoxic intermediate, with the rate of this reaction dependent on the concentration of GSH.

GLUTATHIONE LEVELS AND DRUG RESISTANCE IN HUMAN OVARIAN CANCER CELLS

The GSH levels in human ovarian cancer cell lines and the effects of BSO upon these levels are summarized in table 2 (*22*). The A1847 and A2780 lines were established from ovarian cancer patients before they were treated. These drug-sensitive cell lines were used in the development of drug-resistant variants 1847^{ME}, 2780^{ME}, 2780^{CP}, and 2780^{AD} by stepwise incubation of the sensitive cell lines with increasing concentrations of the specific drugs. Cell lines NIH:OVCAR-3 and 4 were established from patients with ovarian cancer at a time when they were resistant to combination chemotherapy regimens, which included cisplatin, doxorubicin, and cyclophosphamide (*23*). Note that the highest levels of GSH were in these latter cell lines, and these levels were maintained even with continuous passage of these cells in culture for periods longer than 2 years. At nontoxic concentrations, BSO markedly reduced GSH levels in all the tumor cell lines studied (*22–24*). Table 3 presents a summary of the effects of GSH depletion upon the cytotoxicity of doxorubicin, melphalan, and cisplatin in A2780 and its drug-resistant variants (*23*). The dose of drug required to decrease clonogenic cell survival by 50% was reduced by exposure to BSO 1.5- to 11-fold (dose modification factor) when all combinations of drugs and cell lines were considered. The greatest alteration of cytotoxicity occurred in the relatively drug-sensitive A2780 parent cell line, which has a comparatively low baseline GSH concentration. Thus BSO acts as a chemotherapeutic agent sensitizer in contrast to agents, such as verapamil, which act primarily to reverse drug-induced resistance to vinca alkaloids and anthracyclines, but generally have less modifying capacity in sensitive cells (*4*).

In addition to increasing the cytotoxicity of agents in cell lines with drug-induced resistance in vitro, lowering of GSH with BSO potentiated the activity of melphalan in the NIH:OVCAR cell lines derived from patients who had been treated with combination chemotherapy (fig. 1). At a nontoxic concentration, BSO produces a dose-modifying factor of melphalan of 3 in this cell line (*25*).

To determine whether the reduction of GSH was of potential clinical utility, we examined the effects in vivo of BSO upon melphalan cytotoxicity in nude mice bearing an ascites tumor. We had previously adapted NIH:OVCAR-3 for intraperitoneal growth in athymic mice (*26*). In an in vivo model, in which the mice die of massive ascites and intra-abdominal carcinomatosis, BSO administered in the drinking water for 5 days decreased levels in tumor cells by 96%, compared with a reduction in GSH levels of 79% and 86% in the bone marrow and gastrointestinal mucosa, respectively (*25*). In the range of LD_{10} doses, BSO did not produce an increase in the lethality of melphalan in non-tumor-bearing nude mice. However, in tumor-bearing nude mice, a single melphalan treatment of 5 mg/kg following GSH depletion with L-BSO was markedly superior to treatment with melphalan alone and produced a 72% increase in median survival time. Furthermore, BSO treatment of human bone marrow cells prior to melphalan exposure had little effect on melphalan toxicity as assessed in the colony-forming unit–granulocyte–macrophage assay. These results suggest that BSO preferentially enhances the cytotoxic effects of alkylating agents against human ovarian cancer cells and thus overcomes acquired resistance as well as potentiates cytotoxicity in drug-sensitive cells.

GLUTATHIONE ELEVATIONS AND RADIATION SURVIVAL PARAMETERS IN HUMAN OVARIAN CANCER

Radiation survival curves for A2780 and the variant drug-resistant cells are shown in figure 2. The D_0 for A2780 is 101, whereas the $\bar{n}$ is 1.40 (*27*). In contrast, 2780^{ME} has a D_0 of 146 and an $\bar{n}$ of 2.12, and 2780^{CP} has a D_0 of 187 and an $\bar{n}$ of 1.62. This increase in resistance to radiation in 2780^{CP} and 2780^{ME} is statistically significant. In contrast, 2780^{AD} has a D_0 of 111 with an $\bar{n}$ of 1.48, which is not statistically different from those of the parent cell line A2780.

TABLE 2.—Effect of BSO on GSH levels in human ovarian cancer cell lines

GSH, nmol/10^6 cells	Cell lines					
	A2780	2780^{AD}	2780^{ME}	2780^{CP}	NIH:OVCAR-3	NIH:OVCAR-4
Before BSO	1.9	2.9	4.8	6.1	15.1	12.1
After BSO[a]	0.6	0.6	0.7	0.7	2.3	2.1
Percent reduction in GSH	70	78	86	89	85	83

[a] We administered 15–25 μM BSO for 48 hr.

TABLE 3.—Dose modification for doxorubicin, melphalan, and cisplatin in human ovarian cancer cell lines following BSO depletion of GSH

Cell line	Dose modification factor[a]		
	Doxorubicin	Melphalan	Cisplatin
A2780	11.4	6.8	4.3
2780^{ME}	1.5	3.4	1.8
2780^{CP}	2.5	6.3	3.2
2780^{AD}	1.5–5	6.0	3.8

[a] Dose modification factor=(IC_{50} in absence of BSO)÷(IC_{50} in presence of BSO).

Depletion of intracellular GSH by 48-hour monolayer exposure to BSO followed by maintenance of the GSH depleted state in the presence of BSO in the cloning media both during radiation and for 4 days thereafter led to a marked sensitization in 2780^{ME} to irradiation. The D_0 decreased from 143 to 95. Similar treatment of 2780^{CP} resulted in less sensitization to irradiation with a D_0 changing from 183 to 134. However, no significant changes in $\bar{n}$ values were seen in these lines.

DNA REPAIR IN DRUG-RESISTANT CELL LINES

The observation that induced resistance to cisplatin and melphalan is accompanied by cross-resistance to other agents, which cause DNA damage, as well as irradiation, suggests that a common repair mechanism is responsible for the primary resistance to alkylating agents, cisplatin, and to irradiation. Previously, indirect evidence for the role of GSH in repair of irradiation toxicity has been provided by studies which demonstrated that a GSH ester administered to lymphoid cells after irradiation was able to decrease markedly the lethality of irradiation (*28*). We directly evaluated DNA repair as a possible mechanism of cisplatin and melphalan resistance in the cell lines with induced resistance to these agents. Cell lines were screened for their capacity to perform UDS with exposure to melphalan or cisplatin (*29*). In the absence of semiconservative DNA synthesis, [^{3}H]dThd incorporation into acid-precipitable DNA can be taken as a measure of repair of induced damage to DNA. We used hydroxyurea to inhibit semiconservative DNA synthesis and, after 1 hour, melphalan or cisplatin and 5 μCi [^{3}H]dThd/ml were added. After a 3-hour incubation, dishes were washed with ice-cold phosphate-buffered saline and followed by trichloroacetic acid. The DNA was extracted and [^{3}H]dThd incorporation into DNA was determined in a liquid scintillation counter. The UDS activity in the cell lines was expressed as the ratio of disintegrations per minute per microgram of DNA in drug-treated cells to incorporation of [^{3}H]dThd in DNA of untreated control cells. As can be seen in figure 3, melphalan and cisplatin treatment of a cisplatin-resistant cell line was associated with dose-dependent increases in UDS activity, whereas the sensitive cell line showed essentially no capacity to repair DNA damage caused by these drugs.

DISCUSSION

These results demonstrated that human ovarian cancer cell lines with induced resistance to melphalan and cisplatin have increased levels of GSH. The development of resistance to the primary agent is associated with the development of cross-resistance to other drugs as well as to radiation. Lowering of GSH levels with BSO potentiates the cytotoxicity of cisplatin and melphalan in vitro and also reverses the cross-resistance to radiation in human ovarian cancer cell lines with primary induced resistance to melpha-

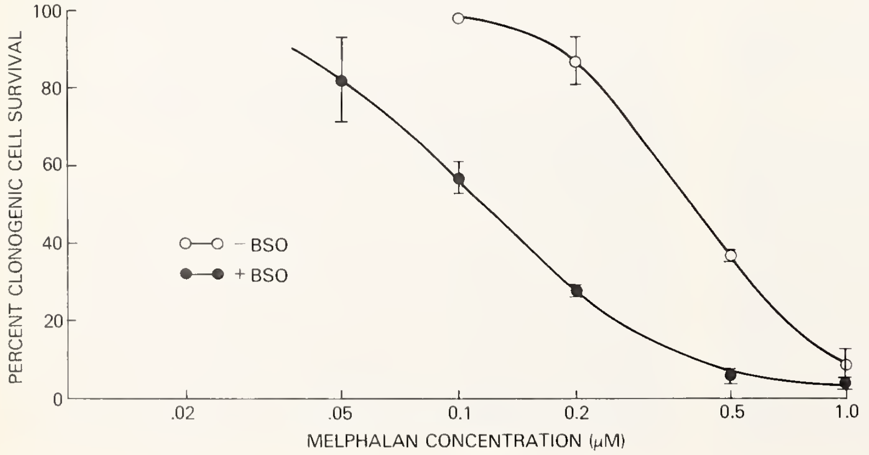


FIGURE 1.—Dose-response curve for NIH: OVCAR-3 to melphalan ± BSO. Lowering GSH levels with BSO increased the cytotoxicity of melphalan threefold (ratio of IC_{50} without BSO to IC_{50} with BSO).

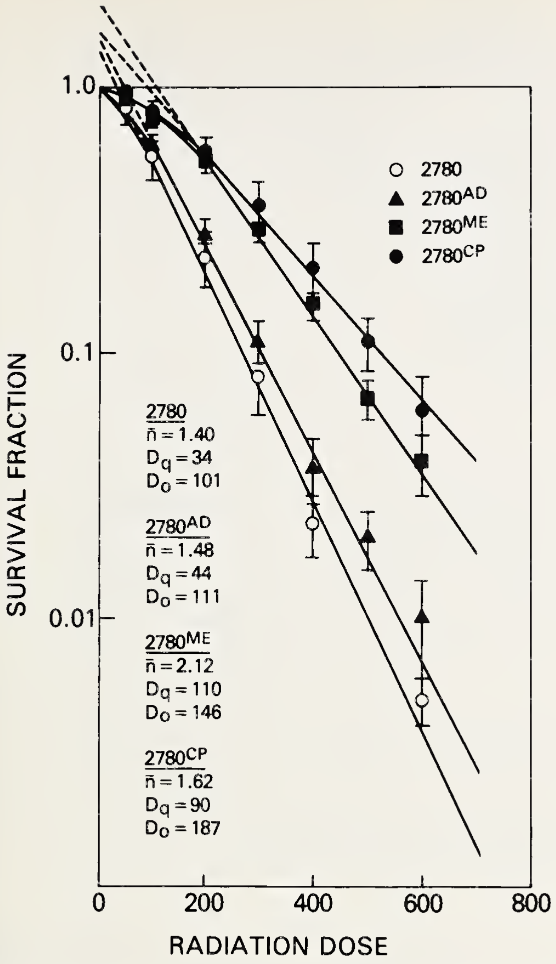


FIGURE 2.—Radiation (dose in rad) survival curves for the drug-sensitive A2780 cell line and its drug-resistant variants.

lan and cisplatin. Furthermore, lowering of GSH levels in an in vivo model of human ovarian cancer markedly potentiates the cytotoxicity of melphalan and leads to prolonged survival. On the basis of these results and those of other studies demonstrating that it potentiates radiation cytotoxicity as well as antineoplastic drug cytotoxicity, BSO has been entered in the Decision Network of the National Cancer Institute for large animal toxicology studies before being used in clinical trials. Initial trials with BSO will be in drug-resistant patients with ovarian cancer, and the effects of BSO upon melphalan cytotoxicity will be evaluated. Although BSO also potentiates the cytotoxicity of cisplatin in vitro, the combination of cisplatin plus BSO is not likely to be of clinical benefit, inasmuch as BSO potentiates the nephrotoxicity of cisplatin. Consequently, it is obvious that the design of clinical trials will require our careful preclinical evaluation of the individual drugs and BSO to determine those drugs in which there appears to be a therapeutic window between efficacy and increased toxicity.

The exact mechanism by which GSH modulates the cytotoxicity of melphalan, cisplatin, and radiation remains to be established (*28–34*). Figure 4 depicts possible sites of action of GSH on melphalan and cisplatin toxicity. Currently, we have no evidence that the melphalan- and cisplatin-resistant ovarian cancer cell lines have a marked decrease in drug accumulation of these agents. However, there is evidence that melphalan-resistant cell lines can detoxify melphalan to the less active dihydroxy melphalan derivative. This reaction may be facilitated by GSH-dependent transferases. Furthermore, it is also possible that GSH can directly interact with either cisplatin or melphalan to produce inactive adducts. Also, GSH may interact with the chromatin structure to decrease the formation of specific DNA cisplatin adducts or decrease the likelihood of cross-link formation following melphalan exposure. Moreover, GSH may be involved in facilitating the repair of specific DNA adducts and melphalan cross-links as well as cross-links produced by radiation. Finally, it is possible that GSH is exerting its effects by interacting at multiple sites in the pathway to cytotoxicity of alkylating agents, cisplatin, and radiation.

Studies are in progress in which investigators are using the more complex density shift methodology to evaluate DNA repair instead of the UDS assay. In addition, for an evaluation of the functional effect of repair upon drug cytotoxicity, we are conducting experiments with inhibitors of DNA repair enzymes to determine the effect upon drug cytotoxicity. These studies should help clarify the overall importance of DNA repair as a mechanism of anticancer drug resistance. Thus it seems clear that more than one mechanism is involved in pleiotropic drug resistance. Although decreased accumulation of drugs may be an important factor with some tumors and some anticancer agents (particularly the natural products), it is apparent that a different mechanism exists for the cross-resistance between cisplatin, melphalan, and radiation that is responsible for the multidrug resistance phenotype observed in

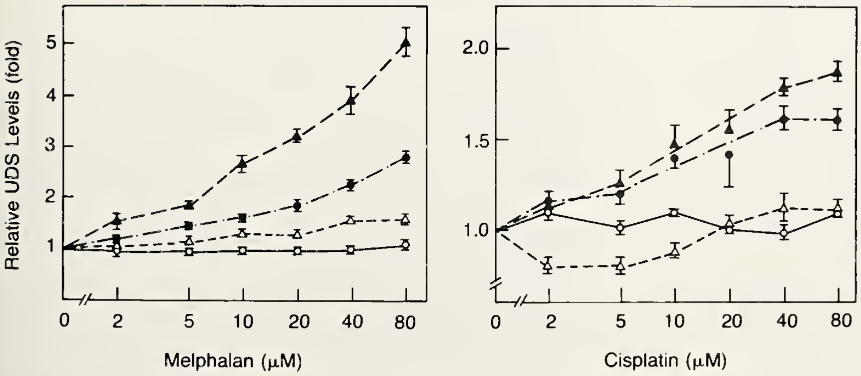


FIGURE 3.—Repair of DNA in cisplatin- and melphalan-resistant human ovarian cancer cell lines. Compared with the sensitive cell line, increase in DNA repair was significant (measured as UDS) in melphalan- and cisplatin- but not doxorubicin-resistant cell lines.

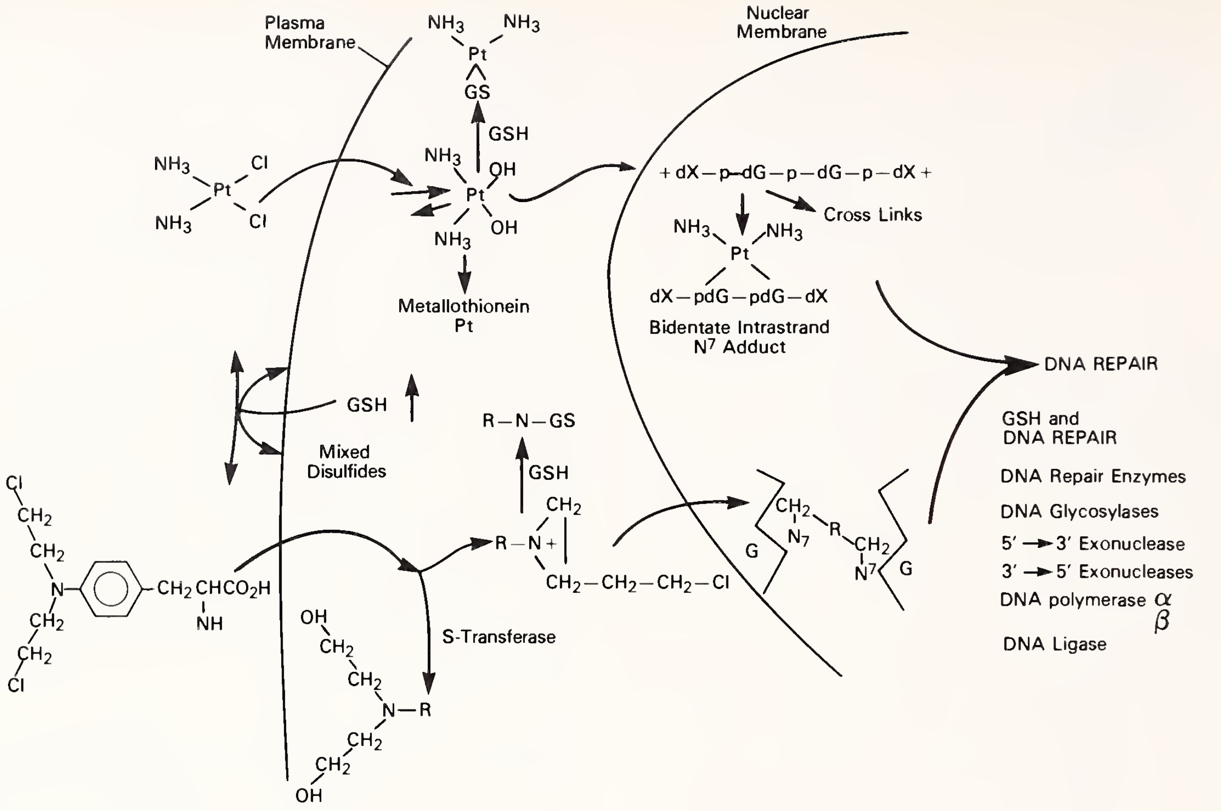


Figure 4.—Possible sites of GSH modulation of melphalan and cisplatin cytotoxicity.

many solid tumors such as ovarian cancer. As our understanding of the specific mechanisms involved in drug cytotoxicity and in the mechanism of resistance increases, it not only demonstrates the complexity of interrelated biochemical processes, but also identifies multiple sites where there is potential for our intervening with clinically useful agents to reverse the multidrug-resistant phenotype.

REFERENCES

(*1*) Dembo A: Radiotherapeutic management of ovarian cancer. Semin Oncol 11:238–250, 1984.

(*2*) Hoskins WJ, Lichter AS, Whittington R, et al: Whole abdominal and pelvic irradiation in patients with minimal disease at second-look surgical reassessment for ovarian carcinoma. Gynecol Oncol 20:271–280, 1985.

(*3*) Malcolm AV, Hainsworth JD, Johnson DH, et al: Advanced minimal residual ovarian carcinoma: Abdominopelvic irradiation following combination chemotherapy. Proc ASCO 2:151, 1983.

(*4*) Ozols RF, Cowan K: New aspects of clinical drug resistance. The role of gene amplification and the reversal of resistance in drug refractory cancer. *In* Important Advances in Oncology (DeVita VT Jr, Hellman S, Rosenberg S, eds). Philadelphia: Lippincott, 1986, pp 129–158.

(*5*) Curt GA, Clendeninn NJ, Chabner BA: Drug resistance in cancer. Cancer Treat Rep 68:87–99, 1984.

(*6*) Juliano RL, Ling V: A surface glycoprotein modulating drug permeability in Chinese hamster ovary cells mutants. Biochim Biophys Acta 455:152–162, 1976.

(*7*) Debenham PG, Kartner N, Siminovitch L, et al: DNA-mediated transfer of multiple drug resistance and plasma membrane glycoprotein expression. Mol Cell Biol 2:881–889, 1982.

(*8*) Ling V: Genetic basis of drug resistance in mammalian cells. *In* Drugs and Hormone Resistance in Neoplasia (Bruchovsky N, Goldie JH, eds). Boca Raton, FL: CRC Press, 1982, pp 1–19.

(*9*) Roninson IB: Detection and mapping of homologous, repeated and amplified DNA sequences by DNA renaturation in agarose gels. Nucleic Acids Res 11:5413–5431, 1983.

(*10*) Riordan JR, Deuchars K, Kartner N, et al: Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. Nature 316:817–819, 1985.

(*11*) Bliek AMV, Velde-Koerts TV, Ling V, et al: Overexpression and amplification of 5 genes in a multidrug-resistant Chinese hamster ovary cell line. Mol Cell Biol 6:1671–1678, 1986.

(*12*) Bell DR, Gerlach JH, Kartner W, et al: Detection of P-glycoprotein in ovarian cancer: A molecular marker associated with multidrug resistance. J Clin Oncol 3:311–315, 1985.

(*13*) Rogan AM, Hamilton TC, Young RC, et al: Reversal of adriamycin resistance by verapamil in human ovarian cancer. Science 224:994–996, 1984.

(*14*) Louie KG, Hamilton TC, Winker MA, et al: Adriamycin accumulation and metabolism in adriamycin-sensitive and resistant human ovarian cancer cell lines. Biochem Pharmacol 35:467–472, 1986.

(*15*) Hubbard SM, Barkes P, Young RC: Adriamycin therapy for advanced ovarian cancer recurrent after chemotherapy. Cancer Treat Rep 62:1375–1377, 1978.

(*16*) Arrick BA, Nathan CF: Glutathione metabolism as a determinant of therapeutic efficacy: A review. Cancer Res 44:4224–4232, 1984.

(*17*) Relle-Somfai S, Suzukake K, Vistica BP, et al: Reduction in cellular glutathione by buthionine sulfoximine and sensitization of murine tumor cells resistant to L-phenylalanine mustard. Biochem Pharmacol 33:485–499, 1984.

(*18*) Ball CR, Connors TA, Double JA, et al: Comparison of nitrogen mustard sensitive and resistant Yoshida sarcoma. Int J Cancer 1:319–327, 1966.

(*19*) Griffith OW: Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. J Biol Chem 257:13704–13712, 1982.

(*20*) Griffith OW, Meister A: Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (*S-n*-butyl homocysteine sulfoximine). J Biol Chem 253:7558–7560, 1979.

(*21*) Suzukake K, Petro BJ, Vistica DL: Dechlorination of L-phenylalanine mustard by sensitive and resistant tumor cells and its relationship to intracellular glutathione levels. Biochem Pharmacol 32:165–167, 1983.

(*22*) Hamilton TC, Winker MA, Louie KG, et al: Augmentation of Adriamycin, melphalan and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian cancer cell lines by buthionine sulfoximine-mediated glutathione depletion. Biochem Pharmacol 34:2583–2586, 1985.

(*23*) Hamilton TC, Young RC, McKoy WM, et al: Characterization of a human ovarian carcinoma cell line (NIH: OVCAR-3) with androgen and estrogen receptors. Cancer Res 43:5379–5389, 1983.

(*24*) Green JA, Vistica DT, Young RC, et al: Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. Cancer Res 44:5427–5431, 1984.

(*25*) Ozols RF, Louie KG, Plowman J, et al: Enhanced alkylating agent cytotoxicity in human ovarian cancer in vitro and in tumor-bearing nude mice by buthionine sulfoximine depletion of glutathione. Biochem Pharmacol 36:147–153, 1987.

(*26*) Hamilton TC, Young RC, Louie KG, et al: Characterization of a xenograft model of human ovarian carcinoma which produces ascites and intraabdominal carcinomatosis. Cancer Res 44:5286–5290, 1984.

(*27*) Louie KG, Behrens BC, Kinsella TJ, et al: Radiation survival parameters of antineoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. Cancer Res 45:2110–2115, 1985.

(*28*) Wellner VP, Anderson ME, Puri RN, et al: Radioprotection by glutathione ester: Transport of glutathione ester into human lymphoid cells and fibroblasts. Proc Natl Acad Sci USA 81:4732–4735, 1984.

(*29*) Behrens BC, Hamilton TC, Masuda H, et al: Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Cancer Res 47:414–418, 1987.

(*30*) Babson JR, Abell NS, Reed DJ: Protective role of the glutathione redox cycle against Adriamycin-mediated toxicity in isolated hepatocytes. Biochem Pharmacol 30:2299–2304, 1981.

(*31*) Meister A: Selective modification of glutathione metabolism. Science 220:472–477, 1983.

(*32*) Biaglow JE, Varnes ME: The role of thiols in cellular response to radiation and drugs. Radiat Res 95:437–455, 1983.

(*33*) Biaglow JE, Clark ER, Morsequadio MM, et al: Nonprotein thiols and the radiation response of A549 human lung carcinoma cells. Int J Radiat Biol 44:489–495, 1983.

(*34*) Bump EA, Yu NY, Brown MJ: Radiosensitization of hypoxic tumor cells by depletion of intracellular glutathione. Science 217:544–545, 1982.

Inhibition of Radiation Dose-rate-sparing Effects in Human Tumor Cells by 3-Aminobenzamide [1]

Lloyd R. Kelland,* Lynne Burgess, and G. Gordon Steel[2,3]

ABSTRACT—The capacity of two recently established human tumor cell lines (a germ cell carcinoma of the testis and a squamous cell cervical carcinoma) to repair radiation damage has been determined by assessing in vitro cell survival over the dose-rate range of 150–1.6 cGy/minute. Survival after irradiation at a high dose rate (150 cGy/min) showed that the cervical carcinoma was more radioresistant. A dose-sparing effect was observed at low dose rates (3.2 and 1.6 cGy/min), compared to the high dose rate, to a similar extent in both cell lines. In addition, 3-aminobenzamide added 2 hours prior to and removed 18 hours after irradiation caused a small but similar increase in cell kill with the high dose rate in both cell lines. It also steepened the survival curves at low dose rates. Since the survival curves obtained at 1.6 and 3.2 cGy/minute help to define a dominant component of the initial slope of the acute curve, we conclude that 3-aminobenzamide can act as a modifier of this component.—NCI Monogr 6:167–171, 1988.

The reviews of Fertil and Malaise (*1*) and Deacon et al. (*2*) have implied that there is a positive correlation between the steepness of the initial part of the survival curve for in vitro radiation (e.g., surviving fraction at 2 Gy) in human tumor cells and the likelihood of achieving local tumor control after radiotherapy. This has placed emphasis on the need to investigate, compare, and moreover, manipulate the initial slopes of the survival curves for different human tumors. One means of achieving such a comparison is to lower the radiation dose rate to a level at which maximal repair is allowed without the occurrence of repopulation or cell-cycle progression. From an analysis of published data on dose-rate dependence, an optimal dose rate was found to be 1–5 cGy/minute (*3*). This approach is supported by current mathematical models for radiation dose-rate dependence (*4,5*) that are based on the concepts of reparable and irreparable radiation damage, which propose that the initial slope of the cell-survival curve represents the fully repaired state of the cells.

Here we report studies on dose-rate effects over the range of 150–1.6 cGy/minute in two recently established human tumor cell lines, a germ cell tumor of the testis and a squamous cell carcinoma of the cervix. In addition, an attempt to modify the initial slope of the radiation cell-survival curve in these tumor lines has been made by the use of nontoxic doses of 3-AB, an agent thought to exert its effects by competing for NAD as the substrate for ADP-ribosylation (*6*). This process leads to the synthesis of poly(ADP-ribose) and is believed to be involved in eukaryotic DNA excision repair (*7–9*).

MATERIALS AND METHODS

Cell lines.—Two recently established human tumor cell lines have been used in this investigation: GCT27J, a germ cell tumor of the testis (established and donated by Dr. M. Pera of this department), and HX156C, a newly derived squamous cell carcinoma of the cervix. Cells were maintained as monolayer cultures in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (Imperial Laboratories), 10^5 units of penicillin/liter, 100 mg of streptomycin/liter, 2 m*M* glutamine, and 0.4 mg of hydrocortisone/liter in an atmosphere of 5% CO_2, 5% O_2, 90% N_2. Cells were used from passages 10 to 30; cloning efficiencies were 30%–40% for GCT27J and 20%–35% for HX156C. Both lines were tumorigenic in nude mice. Cells were periodically checked for mycoplasma contamination by staining with Hoechst 33528 dye and examination under a fluorescent microscope. Comprehensive details of the cell lines will be published separately.

Preparation procedure.—When monolayer cultures were just confluent, a single-cell suspension was prepared by disaggregating the monolayer with 0.05% trypsin in 0.02% Versene and gentle filtration through a 20-μm polyester mesh. Viable cells were counted using lissamine green dye exclusion and phase-contrast microscopy. Prior to irradiation, cells were prepared in a standardized manner to minimize cell-cycle and disaggregation effects. Cells were plated at the appropriate density on three replicate 60-mm tissue culture dishes (Nunc Products) per experimental point; each plate contained 5 ml of Dulbecco's Modified Eagle Medium plus 15% fetal calf serum and 2×10^5 heavily irradiated (200 Gy) Swiss mouse 3T3 feeder cells. When it was indicated, 3-AB (Sigma Chemical Co.) dissolved in phosphate-buffered saline was added. Plates for each dose point and for control unirradiated cells were then placed in

---

ABBREVIATIONS: 3-AB = 3-aminobenzamide; LPL = lethal-potentially lethal; $D_{0.01}$ = dose required for 1% survival at low or high dose rate; DRF = dose reduction factor (ratio of radiation doses that give 1% survival at 150, 3.2, and 1.6 cGy/min); DER = dose enhancement ratio (ratio of radiation doses that give 1% survival with and without addition of 3-AB).

---

[1] Supported by Public Health Service grant CA-26059 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Radiotherapy Research Unit, Institute of Cancer Research, Royal Cancer Hospital, Sutton, Surrey, United Kingdom.

[3] We thank Dr. Julian Delic and Dr. Otto Schlappack for helpful discussion and Mrs. S. Stockbridge and Miss R. Couch for typing the manuscript.

* *Reprint requests:* Lloyd R. Kelland, Ph.D., Radiotherapy Research Unit, Institute of Cancer Research, Royal Cancer Hospital, Clifton Ave., Sutton, Surrey SM2 5PX, United Kingdom.

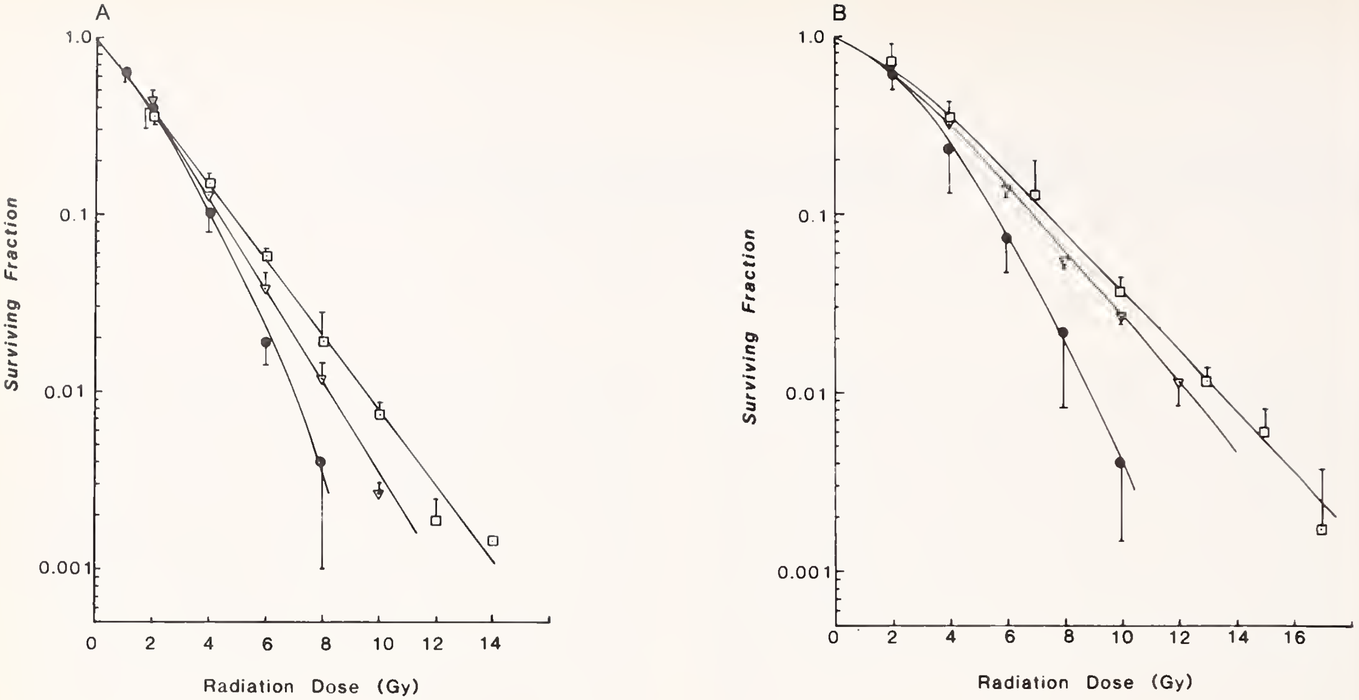


FIGURE 1.—Survival of GCT27J germ cell carcinoma cells (A) or HX156C squamous cell cervical carcinoma cells (B) irradiated in suspension at high dose rate of 150 cGy/min (●) and at continuous low dose rates of 3.2 cGy/min (▽) or 1.6 cGy/min (⊡). Full lines are calculated by simultaneous fitting of all 3 data sets using Thames incomplete repair model (*11*).

plastic boxes, sealed with Sellotape, and gassed for 30 minutes at a flow rate of 2.0 liters/minute with a mixture of 90% N_2, 5% CO_2, 5% O_2. No alterations in pH were detected throughout the experiments. Cells were then incubated for 90 minutes at 37° C before irradiation.

Irradiation procedure.—All irradiation was performed using a ^{60}Co source within a dual-source (2,000/100 Ci) tele-cobalt irradiation room. The larger 2,000-Ci source was used for high-dose-rate irradiation (150 cGy/min), while the smaller source allowed a dose-rate range of 1–30 cGy/minute to be used with identical geometry. Regular dosimetric determinations were made using an Ionex type 2500/3 dosimeter. In all cases, a high-dose-rate survival curve (150 cGy/min) was determined on the same day and on the same original cell suspension used for data at 3.2 or 1.6 cGy/minute. Throughout irradiation, cells were held at 37° C in a Perspex temperature-controlled irradiation box. Immediately after irradiation, the boxes were removed to a 37° C incubator until completion of the experiment.

Postirradiation procedure.—After irradiation, plates were incubated for 12 days for GCT27J or 17 days for HX156C at 37° C in an atmosphere of 90% N_2, 5% O_2, 5% CO_2 in a gassing incubator (Queue Systems, Camlab). Plates were then washed with phosphate-buffered saline and stained for 15 minutes using 0.5% methylene blue in 50% methanol–50% water; colonies >50 cells were counted by eye.

Statistical analysis.—Points represent the mean ± 1 SD of at least three experiments. Where appropriate, curves were fitted using the multitarget and linear quadratic models (*10*). Data for dose-rate dependence were fitted using the "incomplete repair" model of Thames (*11*). We have also made use of the LPL model of Curtis (*5*).

RESULTS

Dose-rate Dependence

Figure 1A shows the effect of dose rate on radiosensitivity for GCT27J, with survival curves determined at 150, 3.2, and 1.6 cGy/minute. An excellent fit to the Thames incomplete repair model for dose-rate dependence is obtained. The acute curve is shouldered, and the parameters ± SE of a fitted multitarget equation are $D_0 = 1.08 \pm 0.05$ and $n = 4.28 \pm 1.23$. The α and β values are given in table 1. A dose-sparing effect was observed at a dose rate of 3.2 cGy/minute, with additional sparing at 1.6 cGy/minute. As a measure of the sparing effect, the ratio of $D_{0.01}$ values has been calculated. This DRF reflects changes both in slope and shoulder size. From figure 1A, we obtain DRF values at dose rates of 3.2 and 1.6 cGy/minute of 1.22 ± 0.03 and 1.38 ± 0.06, respectively.

Dose-rate dependence data for HX156C are shown in figure 1B. Again, the fit to the Thames incomplete repair model is excellent, and the acute curve is shouldered with fitted multitarget equation parameters of $D_0 = 1.54 \pm 0.03$ and $n = 4.95 \pm 0.22$. A substantial dose-sparing effect was observed at a dose rate of 3.2 cGy/minute (DRF = 1.39 ± 0.02), with some additional sparing at a dose rate of 1.6 cGy/minute (DRF = 1.49 ± 0.03).

Effect of 3-Aminobenzamide on Radiosensitivity

Preliminary experiments were performed to determine the maximum usable concentration of 3-AB without causing significant cell death. Both cell lines were exposed to varying concentrations of 3-AB for 48 hours, a time in excess of any exposure time used in the low-dose-rate

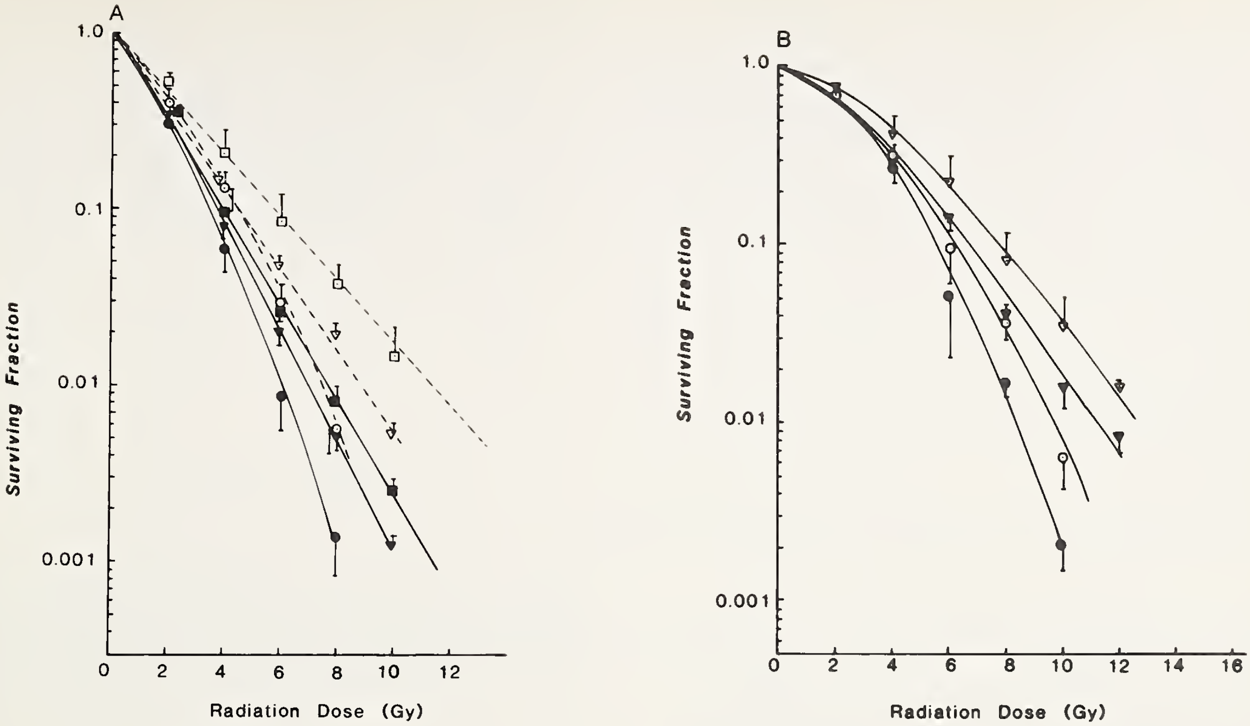


FIGURE 2.—Survival of GCT27J germ cell carcinoma cells (A) or HX156C squamous cell cervical carcinoma cells (B) irradiated in suspension at high dose rate of 150 cGy/min (⊙, ●) and at continuous low dose rates of 3.2 cGy/min [▽, ▼] or 1.6 cGy/min (⊡, ■), either alone (*open symbols*) or in presence of 3-AB (8 m*M* for GCT27J, 7 m*M* for HX156C) added 2 hr prior to and removed 18 hr after irradiation (*closed symbols*). Full lines have all been fitted using multitarget equation (*10*).

experiments. For example, the durations of exposure to 12 Gy were 9.6 minutes, 6 hours, and 12 hours, respectively, for irradiation at dose rates of 150, 3.2, and 1.6 cGy/minute. From such experiments, a concentration of 8 m*M* 3-AB was chosen for GCT27J and 7 m*M* 3-AB was chosen for HX156C. In addition to causing no significant cell death, these concentrations caused no difference in the morphological characteristics or the growth rates in monolayer of the two cell lines.

To determine the effect of 3-AB on radiosensitivity at high and low dose rates, the agent was added 2 hours prior to and removed 18 hours after irradiation to include the possible preirradiation and postirradiation effects of the agent. In all experiments, two complete survival curves (with and without 3-AB) were determined on the same cell suspension at the dose rate of choice. Figure 2A shows the effect of the presence of 3-AB on GCT27J cells irradiated at the high dose rate of 150 cGy/minute and the low dose rates of 3.2 and 1.6 cGy/minute. The 3-AB caused a small but consistent increase in cell lethality when it was present during high-dose-rate irradiation, at all radiation doses tested. The DER (*3*) was calculated as the ratio of radiation doses to give 1% survival with and without 3-AB and was 1.26 ± 0.01. When 3-AB was present 2 hours prior to continuous low-dose-rate irradiation at 3.2 cGy/minute and was removed 18 hours afterward, a greater effect on cell kill was seen, compared to the effect with high-dose-rate irradiation. An even greater effect was observed at 1.6 cGy/minute. The derived DER values were 1.34 ± 0.06 and 1.47 ± 0.07 for 3.2 and 1.6 cGy/minute, respectively.

The effect of the presence of 3-AB on HX156C cells irradiated at either the high dose rate (150 cGy/min) or the low dose rate of 3.2 cGy/minute is shown in figure 2B. Again, there was a small but consistent increase in cell kill at all doses tested when the agent was present during high-dose-rate irradiation. However, when 3-AB was present during irradiation at 3.2 cGy/minute, although some increase in cell kill was apparent, it was not as large as that observed for the germ cell line. The derived DER values for high-dose-rate irradiation and for low-dose-rate irradiation at 3.2 cGy/minute are 1.16 ± 0.03 and 1.10 ± 0.01, respectively.

DISCUSSION

The data presented in figure 2 indicate that a nontoxic dose of 3-AB added 2 hours prior to and removed 18 hours after irradiation produced a steepening of the dose-response curves for both cell lines at each dose rate. Because the results of biochemical studies currently being carried out are not yet available, we have analyzed the effects of 3-AB in the two lines by making use of the LPL model of cell survival described by Curtis (*5*). This is a four-parameter model in which two of the parameters describe the kinetics of repair and misrepair of potentially lethal damage. We have fitted the data shown in figures 1 and 2 using this model, in the hope of identifying the mechanism by which 3-AB administration alters the parameters of the model. In this, we have not been successful. We had anticipated that changes induced by 3-AB might be attributed to a specific change in the rate constant for repair, but the flexibility of the LPL model is such that this is not

TABLE 1.—Summary of survival curve parameters and DER for 3-AB[a]

	DER					
	GCT27J			HX156C		
	150 cGy/min	3.2 cGy/min	1.6 cGy/min	150 cGy/min	3.2 cGy/min	1.6 cGy/min
Without 3-AB						
α	0.356	0.507	0.403	0.161	0.169	0.176
β	0.043	0.007	0.006	0.037	0.018	0.013
DRF		1.22	1.38		1.39	1.49
With 3-AB						
α	0.525	0.699	0.480	0.162	0.198	ND
β	0.040	0	0.015	0.047	0.020	ND
DER	1.26	1.34	1.47	1.16	1.10	ND
Parameters of incomplete repair model						
α (Gy^{-1})		0.435		0.190		
β (Gy^{-2})		0.0346		0.0322		
$T_{1/2}$ (hr)		0.49		1.90		

[a] α and β are parameters of linear quadratic equation. ND = not determined.

uniquely true. We have therefore used the incomplete repair model of Thames (*11*). This fits the data well, and it has allowed us to generate the plot shown in figure 3. We have followed our earlier practice (*3*) of calculating the dose required at each dose rate to achieve a surviving fraction of 0.01. We call these the $D_{0.01}$ values, and in figure 3, they are plotted against dose rate. Each set of experimental data has been fitted with a linear quadratic equation in order to interpolate the best value for $D_{0.01}$. For the points shown in the figure, we included all of the control data given in figure 1. The curves are calculated on the incomplete repair model, and the parameter values are shown in table 1. These values were obtained by fitting all of the cell-survival data shown in figures 1A or 1B simultaneously with the model. The following conclusions can be drawn from figure 3:

1) In terms of the $D_{0.01}$ parameter, the cervical carcinoma cell line HX156C was less radiosensitive than the testicular tumor cell line GCT27J at all dose rates studied.
2) Treatment with 3-AB during irradiation sensitized the cells at all dose rates. The *vertical arrows* indicate the extent of sensitization. In the GCT27J cell line, there was a tendency for the effect to be greater at the lower dose rates, but this was not the case in the cervical carcinoma line. This is also reflected in the trend of increasing DER values given in table 1. These values were calculated by comparison of the 3-AB data with data from the concurrent controls.

Our derived DER values for high-dose-rate irradiation are similar to those observed by other investigators using a number of different benzamide analogues and mammalian cell lines (*12,13*). However, it should be noted that some studies have shown no enhancement in γ-radiation-induced cell kill by 3-AB (*14*). Among the few published studies involving the dose-sparing effect of low dose rate by 3-AB, there is some conflict. In one study involving radiosensitive L5178Y mouse leukemia cells, there was complete inhibition (*15*), whereas in another study involving the use of human fibroblasts, there was no inhibition (*16*). It has been suggested that these apparent differences in effects of 3-AB on different mammalian cells may be due to the fact that inhibition of poly(ADP-ribose) synthesis is more important in radiosensitive lines such as resting lymphocytes and L5178Y-S cells (*17*). Although the two cell lines studied here did not differ greatly in radiosensitivity, there was a tendency toward greater sensitization in the more sensitive germ cell line.

What are the implications of our data for the modification of the initial slope of the cell survival curve? Although the amount of cell kill at 2 Gy is often too small to be measured with great precision, it can be seen from figure 2A that in the GCT27J cells, the administration of 3-AB did tend to reduce survival at all dose rates. When the data are fitted with the linear quadratic equation, the derived values for α (table 1) are consistently increased by the addition of 3-AB in this cell line. For the cervical carcinoma cell line HX156C, the conclusion is not as clear. The α value is unchanged at the high dose rate but may be slightly increased at 3.2 cGy/minute. Our reason for using the low dose rate in these experiments was to allow the fully repaired component of survival, which dominates the initial slope, to be defined more accurately. The modification that we have seen at low dose rate therefore confirms our hypothesis that 3-AB is a modifier of the initial slope.

From this study, we cannot ascertain the mechanisms by which 3-AB exerts the observed sensitizing effects and the apparent differential sensitization in the two tumor cell lines. However, the evidence that 3-AB exerts its effects via an inhibition of DNA repair is compelling (*7–9*).

The significance for clinical radiotherapy suggested by these sensitizing effects of 3-AB in tumor cells is, as yet, unclear. Until parallel normal tissue studies are conducted, we are uncertain whether any therapeutic gain is achievable. If 3-AB did appear to exert some selectivity in tumors, one potentially useful clinical role suggested from our studies would be as an adjuvant to low-dose-rate brachytherapy as used presently in the radiotherapy of carcinomas of the cervix and head and neck.

REFERENCES

(*1*) FERTIL B, MALAISE EP: Inherent cellular radiosensitivity as a basic concept for human tumor radiotherapy. Int J

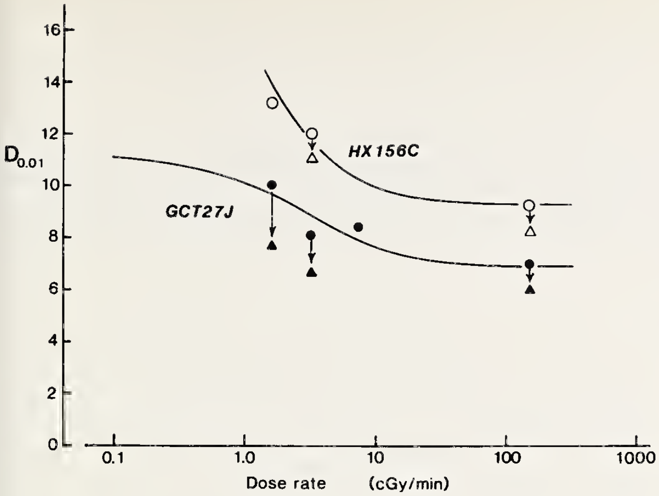


FIGURE 3.—Dose-rate dependence in 2 cell lines and effect of 3-AB. Ordinate indicates dose required for surviving fraction of 0.01 at each dose rate (○ = HX156C; ● = GCT27J). △ and ▲ indicate corresponding values in presence of 3-AB. Full lines calculated on incomplete repair model using parameter values given in table 1. *Vertical arrows* indicate extent of sensitization.

Radiat Oncol Biol Phys 7:621–629, 1981.

(*2*) DEACON J, PECKHAM MJ, STEEL GG: The radioresponsiveness of human tumours and the initial slope of the cell survival curve. Radiother Oncol 2:317–323, 1984.

(*3*) STEEL GG, DOWN JD, PEACOCK JH, et al: Dose-rate effects and the repair of radiation damage. Radiother Oncol 5:321–331, 1986.

(*4*) POHLIT W, HEYDER IR: The shape of dose-survival curves for mammalian cells and repair of potentially lethal damage analysed by hypertonic treatment. Radiat Res 87:613–634, 1981.

(*5*) CURTIS SB: Lethal and potentially lethal lesions induced by radiation—a unified repair model. Radiat Res 106:252–270, 1986.

(*6*) PURNELL MR, WHISH WJD: Novel inhibitors of poly(ADP-ribose) synthetase. Biochem J 185:775–777, 1980.

(*7*) DURCACZ BW, OMIDIJI O, GRAY DA, et al: (ADP-ribose)*n* participates in DNA excision repair. Nature 283:593–596, 1980.

(*8*) SHALL S: ADP-ribose in DNA repair: A new component of DNA excision repair. *In* Advances in Radiation Biology. New York: Academic Press, 1984, pp 1–69.

(*9*) CREISSEN D, SHALL S: Regulation of DNA ligase activity by poly(ADP-ribose). Nature 296:271–272, 1982.

(*10*) MILLAR BC, FIELDEN EM, MILLAR JL: Interpretation of survival-curve data for Chinese hamster cells, line V79, using the multitarget, multitarget with initial slope, and alpha-beta equations. Int J Radiat Biol 33:599–603, 1978.

(*11*) THAMES HD: An 'incomplete-repair' model for survival after fractionated and continuous irradiation. Int J Radiat Biol 47:319–339, 1985.

(*12*) NDUKA N, SKIDMORE CJ, SHALL S: The enhancement of cytotoxicity of *N*-methyl-*N*-nitrosourea and of γ-radiation by inhibitors of poly(ADP-ribose) polymerase. Eur J Biochem 105:525–530, 1980.

(*13*) HUET J, LAVAL F: Influence of poly(ADP-ribose) synthesis inhibitors on the repair of sublethal and potentially lethal damage in γ-irradiated mammalian cells. Int J Radiat Biol 47:655–662, 1985.

(*14*) JAMES MR, LEHMANN AR: Role of poly(adenosine diphosphate ribose) in deoxyribonucleic acid repair in human fibroblasts. Biochemistry 21:4007–4013, 1982.

(*15*) UENO AM, TANAKA O, MATSUDAIRA H: Inhibition of gamma-ray dose-rate effects by D20 and inhibitors of poly(ADP-ribose) synthetase in cultured mammalian L5178Y cells. Radiat Res 98:574–582, 1984.

(*16*) THRAVES P, MOSSMAN KL, BRENNAN T, et al: Radiosensitization of human fibroblasts by 3-aminobenzamide: An inhibitor of poly(ADP-ribosylation). Radiat Res 104:119–127, 1985.

(*17*) BEN-HUR E: Involvement of poly(ADP-ribose) in the radiation response of mammalian cells. Int J Radiat Biol 46:659–671, 1984.

Effects of Methylxanthines on Cell-cycle Redistribution and Sensitization to Killing by Low-dose-rate Radiation [1]

Larry E. Dillehay,* George Chang, and Jerry R. Williams [2,3]

ABSTRACT—The effects of two methylxanthines, caffeine and isobutylmethylxanthine (IBMX), on the response to radiation given at two low dose rates were studied in a human hepatoma cell line, HepG2. These dose rates are in the range to which tumor cells are exposed in radiolabeled immunoglobulin therapy of primary hepatoma. At the higher dose rate (0.3 Gy/hr for 24 hr), the radiation completely inhibited growth, and cells accumulated in the G_2 phase of the cell cycle. Caffeine (1 m*M*), given either continuously or during the last 4 hours of the radiation exposure, counteracted the growth inhibition and G_2 accumulation and enhanced cell killing. The 0.5-m*M* dose of IBMX had little effect on cells. For a lower dose rate and longer exposure period (0.06 Gy/hr for 3 days), the radiation partially inhibited growth and the accumulation of cells in G_2 was small. Continuous, but not short, exposure to caffeine enhanced killing at this dose rate and counteracted the G_2 accumulation. At the lower dose rate, IBMX enhanced the growth inhibition, G_2 accumulation, and killing above that with radiation alone.—NCI Monogr 6:173–176, 1988.

Repopulation by surviving tumor cells during conventional fractionated therapy (2–3 Gy/day, 5 days/wk) may be a source of local control failure only for more rapidly growing tumors (*1,2*). However, for radiotherapy protocols with lower average dose rates, repopulation may more adversely affect the response. One new therapy in which repopulation may be especially important is RIT of primary hepatoma (*3*). In this therapy, the dose rate to the tumor decreases with time after injection of the antibody due to the decay of the radioisotopic label or loss of the antibody, so that part of the dose is delivered after the dose rate may no longer be sufficient to inhibit proliferation. In RIT of primary hepatoma, treatment cycles are separated by the time required for bone marrow recovery. Regrowth of the tumor between cycles, particularly for faster growing alpha-fetoprotein-positive tumors, may be an important factor in failures in achievement of a sustained remission (*3*). Treatments that delay repopulation or increase sensitivity to killing during the low-dose-rate period may be useful in the avoidance of such failures.

Mitchell et al. (*4*) demonstrated that the dose rate that inhibited growth in different cell lines correlates with the RIMD. Therefore, we were interested in a report by Daniel and Oleinick (*5*) that in *Physarum polycephalum* some methylxanthines (e.g., IBMX) could increase RIMD. It is well known that caffeine, a methylxanthine, decreases RIMD (*6*) and increases the killing by acute radiation (*7*). That some methylxanthines increase RIMD and others decrease it may be related to the relative ability of these compounds to inhibit the cleavage of c-AMP and c-GMP (*8*).

In this study, we observed that caffeine and IBMX had the opposite effects on the fraction of cells in the G_2 phase of the cell cycle in human hepatoma cells exposed to low-dose-rate radiation. Caffeine enhanced killing by this type of radiation at the two dose rates studied, whereas IBMX enhanced killing only at the lower dose rate.

MATERIALS AND METHODS

Experiments were conducted with HepG2, a human hepatocellular carcinoma cell line (*9*). We (*10*) described the culture conditions, conditions for irradiating cells with $[^3H]H_2O$, and methods used to determine cell number and clonogenic fraction. Caffeine and IBMX were obtained from Sigma Chemical Co. (St. Louis, MO). For cell-cycle distribution studies, trypsinized cultures were stained with the DNA fluorescent dye ethidium bromide (*11*). Histograms of DNA per cell were then obtained with a Becton Dickinson FACS II flow cytometer (Mountain View, CA). The percentages of cells in the G_1, S, and G_2+M phases of the cell cycle were estimated by a least-squares fit to the histograms by a method similar to that of Dean and Jett (*12*), except that the G_1 and G_2 peaks were fitted to nonequilateral triangles and the S phase to a quadrilateral whose side slopes were determined from the widths of the G_1 and G_2 peaks.

RESULTS

We (*10*) had previously established that for HepG2 cells a dose rate of about 0.08 Gy/hour would reduce the growth rate (over a 2-day period) by 50%. Two combinations of dose rate and exposure period were used in the present studies. We chose a 3-day exposure at 0.06 Gy/hour to represent the situation in RIT in which the radiation can no longer inhibit proliferation, but the dose rate is one at which significant killing would be produced. A 1-day exposure at 0.3 Gy/hour represented the situation at earlier times during a treatment cycle when the dose rate would be higher than that required to inhibit proliferation.

Figure 1 shows the results from an experiment in which

ABBREVIATIONS: RIT = radiolabeled immunoglobulin therapy; RIMD = length of the mitotic delay in each line after acute radiation; IBMX = isobutylmethylxanthine.

[1] Supported by Public Health Service grant CA-06973 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Radiobiology Laboratory, Oncology Center, The Johns Hopkins Hospital, Baltimore, MD.

[3] We thank Linda Nordin for her assistance with the flow cytometer.

* *Reprint requests:* Larry E. Dillehay, Ph.D., Radiobiology Laboratory, Rm. 2-121, Oncology Center, The Johns Hopkins Hospital, 600 N. Wolfe St., Baltimore, MD 21205.

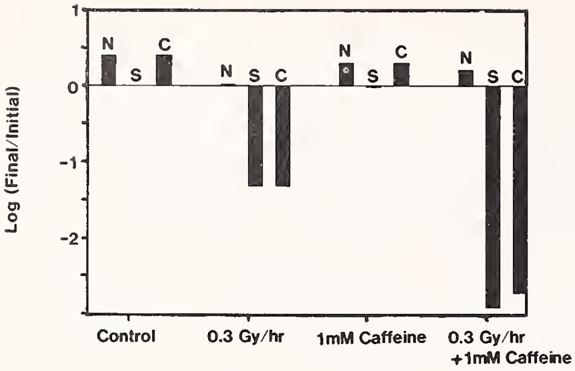


FIGURE 1.—Effects of 24-hr treatments with 0.3 Gy/hr radiation and/or 1 m*M* caffeine on cell number (N), surviving (clonogenic) fraction (S), and total number of clonogenic cells (C). Each parameter was measured immediately before (initial) and after (final) treatments. Logarithm of the ratio of final to initial values is represented by length of bar.

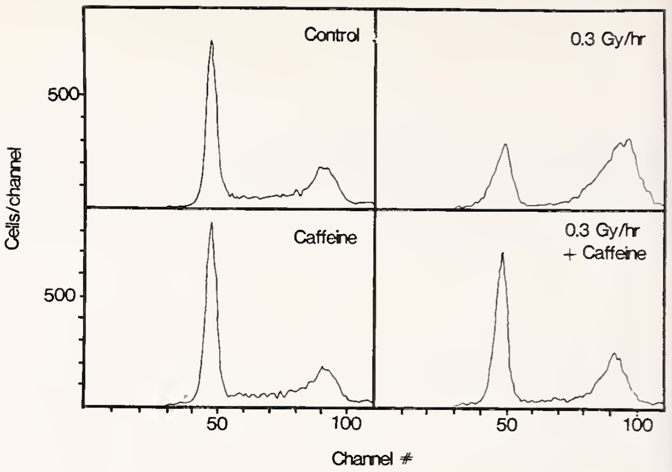


FIGURE 2.—Distribution of DNA/cell after 24 hr with treatment indicated as determined by flow cytometry. Peak centered at channel 48 represents G_1 cells, and peak centered at channel 92 represents $G_2 + M$ cells.

the effects of 0.3 Gy/hour and caffeine were studied in a 1-day exposure. In each experiment in these studies, cell numbers in duplicate cultures were determined at the beginning and end of the treatments. The logarithm of the ratio of final to initial cell numbers for each treatment is shown by the length of the bar marked "N." The fraction of cells able to form colonies was also determined at the end of each treatment and divided by the same fraction for untreated cultures. The logarithm of this ratio (negative for cytotoxic treatments) is represented by the bar marked "S" (for surviving fraction). From these 2 values, the ratio of the total number of clonogenic cells at the end of treatment to the number at the beginning of the treatment was calculated, and the logarithm of this ratio is indicated by the bar marked "C" (for clonogenic cells). A bar extending below the zero line indicates that there were fewer clonogenic cells at the end of the treatment than were present before. Exposure to 0.3 Gy/hour completely inhibited growth during the exposure period and resulted in a decrease in surviving fraction of more than 1 log. Caffeine (1 m*M*) inhibited growth moderately but was not toxic. When caffeine was combined with 0.3 Gy/hour, much less growth inhibition occurred, and cell killing was much greater than with the radiation alone.

In the same experiment, we also took samples at the end of the treatments to determine cell-cycle distribution from flow cytometric measurements of DNA per cell (fig. 2). There was a large increase in the fraction of cells in $G_2 + M$ for 0.3 Gy/hour. Caffeine alone had little effect on cell-cycle distribution. When caffeine and radiation were used, the cell-cycle distribution was more like that of control cultures than that of cultures that were treated with radiation alone.

Table 1 shows results for other treatments in this and another experiment. The IBMX at 0.5 m*M* inhibited growth moderately for 1- or 3-day treatments, as did caffeine for a 3-day treatment, but none was toxic. Table 2 contains a summary of the effects of chemical treatments in modifying the growth inhibition, cytotoxicity, and accumulation in $G_2 + M$ produced by radiation at the two dose rates. At both dose rates, continuous exposure to caffeine produced a large increase in cell killing and counteracted the accumulation in $G_2 + M$. A short exposure to caffeine also reversed the $G_2 + M$ accumulation and enhanced cell killing at the higher dose rate but did not affect cell killing at the lower dose rate. Although at the lower dose rate, IBMX enhanced the G_2 accumulation above that for radiation alone, and more growth inhibition and cytotoxicity were noted, it had little or no effect on these parameters at the higher dose rate.

DISCUSSION

These results indicate that methylxanthines or other compounds that modify accumulation in G_2 might be useful in increasing the destruction of cycling tumor cells in low-dose-rate radiotherapies, such as RIT.

Busse et al. (*7*) observed in HeLa cells that, for irradiation in any part of the cell cycle, a large increase in killing occurred if they were exposed to 1 m*M* caffeine when they had progressed to G_2. When cells arrested in G_2 following acute irradiation are treated with caffeine, they immediately progress to mitosis (*13*). The increased killing is presumably due to the decreased time for repair before the damage becomes unrepairable. In a similar manner, the large increase in killing we observed at 0.3 Gy/hour with caffeine could be due to decreased delay in G_2 (as evidenced by the reduced $G_2 + M$ peak) of radiation-damaged cells. However, because the exposure period (24 hr) was similar to the doubling time for untreated cultures (24–28 hr), time for all cells to progress to G_2 may have been insufficient; other effects of caffeine could be responsible for increased cytotoxicity. A higher concentration (4 m*M*) of caffeine after acute irradiation has been found to inhibit repair of potentially lethal damage in HeLa cells (*14*). However, the observation that a 4-hour exposure to caffeine at the end of the 0.3-Gy/hour exposure produced a considerable increase in cytotoxicity while it eliminated the G_2 accumulation argues that the elimination of the G_2 delay by caffeine plays an important role in the increased cytotoxicity.

Caffeine also sensitizes cells to killing by alkylating agents when given after treatment (*15,16*). However, in these in vitro studies, the 1 or 2 m*M* caffeine had to be

Table 1.—Effects of various treatments on cell number, clonogenic fraction, and cell-cycle distribution[a]

Expt No.	Days	Treatment	Log ratio, final/initial: Cell No.	Log ratio, final/initial: Clonogenic fraction	Log ratio, final/initial: Total cells	Percent: G_1	Percent: S	Percent: G_2+M
48	1	Control	0.39	0.00	0.39	46	31	23
		0.3 Gy/hr	0.01	−1.33	−1.32	28	14	58
		0.3 Gy/hr + 1 m*M* caffeine	0.20	−2.91	−2.71	48	22	30
		*0.05 m*M* IBMX	0.24	−0.05	0.19	40	31	29
		*0.3 Gy/hr + 0.5 m*M* IBMX	0.04	−1.07	−1.03	23	18	59
	3	Control	0.88	0.00	0.88	54	26	20
		0.06 Gy/hr	0.61	−0.73	−0.12	49	17	34
		0.5 m*M* IBMX	0.69	0.00	0.69	44	34	22
		0.06 Gy/hr + 0.5 m*M* IBMX	0.43	−1.01	−0.58	32	13	55
		*0.05 m*M* IBMX + 1 m*M* caffeine (last 6 hr)	0.78	−0.03	0.75	44	33	22
		*0.06 Gy/hr + 0.5 m*M* IBMX + 1 m*M* caffeine (last 6 hr)	0.52	−1.16	−0.64	52	20	28
57	1	Control	0.21	0.00	0.21	56	24	21
		0.3 Gy/hr	0.04	−1.26	−1.22	29	9	63
		0.3 Gy/hr + 1 m*M* caffeine	0.14	−2.58	−2.44	63	11	28
		*0.3 Gy/hr + 1 m*M* caffeine (last 4 hr)	0.10	−1.85	−1.75	61	8	32
	3	Control	0.80	0.00	0.80	56	24	21
		0.06 Gy/hr	0.44	−0.79	−0.35	58	10	33
		*1 m*M* caffeine	0.56	−0.08	0.48	55	25	21
		*0.06 Gy/hr + 1 m*M* caffeine	0.49	−1.44	−0.95	61	21	18
		*0.06 Gy/hr + 1 m*M* caffeine (last 4 hr)	0.51	−0.83	−0.32	65	11	25

[a] Conditions marked with *asterisk* were studied only in the experiment shown. Other conditions were studied in at least 2 experiments with similar results.

present at least 12 hours to produce substantial sensitization. Caffeine has a plasma half-life in man of 3 to 7.5 hours (*17*), and toxicity is often observed above a 0.2-m*M* plasma concentration (*18*). It is thus questionable whether an effective, but nontoxic, sensitization with caffeine could be obtained clinically. Because theobromine sensitizes at similar concentrations as caffeine and has similar pharmacokinetics, but appears to be much less toxic, Byfield and associates (*15*) suggested that theobromine was more likely to be clinically useful. We observed considerable sensitization to continuous irradiation with only a short (4-hr) exposure to caffeine; therefore, sensitization in vivo should be possible if a high enough initial plasma level of caffeine or theobromine could be reached. To be effective with RIT, such a treatment would have to be given at a time when the dose rate is such that cycling tumor cells have accumulated in G_2. Because sensitization with short exposures was not observed at the lower dose rate and in RIT the initial dose rates to normal tissues are lower and decay faster than in the tumor (*19,20*), such an approach might result in a therapeutic gain.

As cells pass from early to late G_2, there is a rapid increase in radiosensitivity. Tolmach et al. (*21*) observed that, for HeLa cells blocked in G_2 by a dose of acute radiation, they were initially relatively insensitive to additional killing due to a second acute dose but after 4 hours showed the increased sensitivity characteristic of late G_2 cells. In a similar manner, the prolongation of G_2 in irradiated cells in the presence of IBMX (as evidenced by the increased G_2+M fraction) may be responsible for the increased cell killing we observed at 0.06 Gy/hour, due to cells entering a state in which they are sensitive to subsequent radiation. That IBMX did not increase killing at 0.3 Gy/hour could be due to the radiation alone being sufficient to block progress out of G_2 completely. In addition to the increased cell killing at the lower dose rate, IBMX also increased the growth inhibition compared with radiation alone. In RIT of primary hepatoma, regrowth of the tumor between treatment cycles has been an important mode of treatment failures, particularly in fast-growing alpha-fetoprotein-positive tumors (*3*). Thus increased growth inhibition at low dose rates could contribute to tumor control. Although we have not tested this point, it is likely that agents that increase sensitivity by prolonging G_2 would have to remain at an effective concentration for a prolonged period to be useful. To our knowledge, the only study of the in vivo

Table 2.—Summary of modifications of radiation effects[a]

Treatment: Dose rate, Gy/hr	Treatment: Chemical	Effect modified: Growth inhibition	Effect modified: Cell killing	Effect modified: G_2 accumulation
0.3	IBMX	0	0	0
	Caffeine	− −	+++	− −
	Caffeine (4 hr)	−	+	− −
0.06	IBMX	+	+	++
	Caffeine	0	++	− −
	Caffeine (6 hr)	0	0	−

[a] A plus indicates that the chemical treatment enhanced the effect of the radiation alone, and a minus indicates inhibition of the radiation effect, with the number of symbols indicating the strength of the enhancement or inhibition. A zero indicates that the chemical had little or no effect on the radiation effect.

effects of IBMX suggests that IBMX levels can be sustained at nontoxic levels that are effective in inhibiting tumor growth. Janik et al. (*22*) gave mice ip injections of 20 mg IBMX/kg twice daily for 9 days with little toxicity. Injections of 10 mg IBMX/kg twice daily for 32 days resulted in a decrease in the number of metastases and a delay in the appearance of lung metastasis from a transplantable tumor.

Agents other than methylxanthines that block cells in G_2 might also sensitize cells to killing by low-dose-rate radiation. Lau and Pardee (*16*) reported that nontoxic concentrations of nitrogen mustard produced a large increase in the fraction of cells in G_2. Just as in the case of radiation-induced G_2 block, addition of caffeine greatly increased cell killing. A number of alkylating agents and antimetabolites were also found to produce G_2 arrest and caffeine sensitization, with the period of arrest occurring at various times after treatment with different agents (*16*).

REFERENCES

(*1*) Peters LJ, Withers HR, Thames HD, et al: The problem: Tumor radioresistance in clinical radiotherapy. Int J Radiat Oncol Biol Phys 8:101–107, 1982.

(*2*) Fowler JF: Potential for increasing the differential response between tumors and normal tissues: Can proliferation rate be used? Int J Radiat Oncol Biol Phys 12:641–645, 1986.

(*3*) Order SE, Stillwagon GB, Klein JL, et al: Iodine 131 antiferritin, a new treatment modality in hepatoma: A Radiation Therapy Oncology Group study. J Clin Oncol 3:1573–1582, 1985.

(*4*) Mitchell JB, Bedford JS, Bailey SM: Dose-rate effects in mammalian cells in culture. III. Comparison of cell killing and cell proliferation during continuous irradiation for six different cell lines. Radiat Res 79:737–751, 1979.

(*5*) Daniel JW, Oleinick NL: Cyclic nucleotide responses and radiation-induced mitotic delay in *Physarum polycephalum.* Radiat Res 97:341–351, 1984.

(*6*) Scaife JF: Mitotic G_2 delay induced in synchronized human kidney cells by UV and X-irradiation and its relation to DNA strand breakage, repair and transcription. Cell Tissue Kinet 3:229–242, 1970.

(*7*) Busse PM, Bose SK, Jones RW, et al: The action of caffeine on X-irradiated HeLa cells. III. Enhancement of X-ray-induced killing during G_2 arrest. Radiat Res 76: 292–307, 1978.

(*8*) Daniel JW, Oleinick NL: Participation of elevated levels of cyclic GMP in the recovery from radiation-induced mitotic delay. Int J Radiat Biol 45:78–83, 1984.

(*9*) Knowles B, Howe C, Aden D: Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 209:497–499, 1980.

(*10*) Dillehay LE, Cathers LE: Low-dose-rate radiation sensitivity of a human hepatoma cell line. NCI Monogr 3:43–46, 1987.

(*11*) Vindelov LL: Flow microfluorometric analysis of nuclear DNA in cells from solid tumors and cell suspensions. Virchows Arch [Cell Pathol] 24:227–242, 1977.

(*12*) Dean PN, Jett JH: Mathematical analysis of DNA distributions derived from flow microfluorometry. J Cell Biol 60:523–527, 1974.

(*13*) Walters RA, Curley LR, Tobey RA: Effects of caffeine on radiation-induced phenomena associated with cell-cycle traverse of mammalian cells. Biophys J 14:99–118, 1977.

(*14*) Beetham KL, Tolmach LJ: The action of caffeine on x-irradiated HeLa cells. VII. Evidence that caffeine enhances expression of potentially lethal radiation damage. Radiat Res 100:585–593, 1984.

(*15*) Byfield JE, Murnane J, Ward JF, et al: Mice, men, mustards and methylated xanthines: The potential role of caffeine and related drugs in the sensitization of human tumors to alkylating agents. Br J Cancer 43:669–683, 1981.

(*16*) Lau CC, Pardee AB: Mechanism by which caffeine potentiates lethality of nitrogen mustard. Proc Natl Acad Sci USA 79:2942–2946, 1982.

(*17*) Curatol PW, Robertson D: The health consequences of caffeine. Ann Intern Med 98:641–653, 1983.

(*18*) Rall TW: Central nervous system stimulants: The methylxanthines. *In* Pharmacological Basis of Therapeutics (Goodman Gilman AF, Goodman LS, Rall TW, et al, eds), 7th ed. New York: Macmillan, 1985, pp 589–603.

(*19*) Order SE, Klein JL, Ettinger D, et al: Phase I–II study of radiolabeled antibody integrated in the treatment of primary hepatic malignancies. Int J Radiat Oncol Biol Phys 6:703–710, 1980.

(*20*) Order SE, Klein JL, Leicher PK, et al: ^{90}Yttrium antiferritin—a new therapeutic radiolabeled antibody. Int J Radiat Oncol Biol Phys 12:277–281, 1986.

(*21*) Tolmach LJ, Griffiths TD, Jones RW: Susceptibility of x-ray-arrested HeLa S3 cells to additional arrest. Radiat Res 66:649–654, 1976.

(*22*) Janik P, Assaf A, Bertram JS: Inhibition of growth of primary and metastatic Lewis lung carcinoma cells by the phosphodiesterase inhibitor isobutylmethylxanthine. Cancer Res 40:1950–1954, 1980.

Interactions Between Antitumor Drugs and Radiation in Mammalian Tumor Cell Lines: Differential Drug Responses and Mechanisms of Resistance Following Fractionated X-Irradiation or Continuous Drug Exposure In Vitro

Bridget T. Hill,* Richard D. H. Whelan, Louise K. Hosking, Sharon A. Shellard, Philip Bedford, and Richard B. Lock[1]

ABSTRACT—**Drug-resistant mammalian tumor cell lines have been derived by either fractionated x-irradiation treatment or exposure to vincristine or etoposide (VP-16-213) in vitro. Analyses of the patterns of responses expressed by these differently derived, resistant cell lines have shown variations in responses to a range of antitumor drugs depending upon the agent used to induce resistance. However, *all* treated cell lines express resistance to vincristine and, with one exception, to VP-16-213. Preliminary evidence has indicated that resistance to vincristine in drug-treated cells, but not x-irradiation-treated cells, is associated with impaired vincristine uptake; resistance to VP-16-213 in both differently derived, resistant sublines is associated with a reduction of VP-16-213-induced DNA single-strand breakage; and collateral sensitivity to cisplatin in x-irradiation-treated cells is associated with enhanced drug-induced DNA cross-linking. These data indicate that patterns of responses to antitumor drugs and the mechanisms associated with these altered responses differ depending upon the agent used to induce resistance.—NCI Monogr 6:177-181, 1988.**

Prior radiotherapy or chemotherapy significantly reduces clinical response rates to subsequent chemotherapy in a number of tumor types including: squamous cell carcinoma of the head and neck (*1,2*), breast carcinoma (*3*), ovarian carcinoma (*4*), and small cell lung cancer (*5*). In an effort to identify the basis for these reduced responses, we derived a series of drug-resistant mammalian tumor sublines in vitro either by fractionated x-irradiation treatment or by exposure to specific drugs. We first established their patterns of in vitro drug sensitivities and are now attempting to determine the mechanisms associated with the differential drug responses expressed by these independently derived sublines. Initial experiments were performed with the murine L5178Y lymphoblastoid cell line (*6*), and now we have extended these to include 4 cell lines derived from different human tumor types. Some of the basic growth characteristics of these human tumor cell lines are listed in table 1.

DERIVATION, CHARACTERIZATION, AND EVALUATION OF DRUG RESPONSES OF X-IRRADIATION-TREATED SUBLINES

Derivation of X-irradiation-treated Sublines

Logarithmically growing tumor cell lines were exposed in vitro to an approximate ID_{90} dose of x-irradiation for between 8 and 11 fractions, allowing the cell populations to recover between each fraction, and then they were plated at a lower density. Logarithmically growing L5178Y lymphoblasts received 10 fractions of 2 Gy over 24 weeks, to a total dose of 20 Gy, as described earlier (*6*). By this method, the sublines HN-1/DXR_{11} (*11*), MCF-7/DXR_{10} (*12*), SuSa/DXR_{10}, and RT112/DXR_8 were generated; they had received a total of 50, 60, 30, and 48 Gy, respectively, approximating the clinical dose range used for these human tumor types.

Characterization of X-irradiation-treated Sublines

Full characterization, according to various cytologic, biologic, and kinetic parameters of both the L5178Y parental and DXR_{10} cells (*6*) and the HN-1 parental and DXR_{11} cells (*11*), has been published. None of these properties were shown to differ significantly between the 2 cell lines. We have also now confirmed that the growth characteristics of the x-irradiated sublines of MCF-7, SuSa, and RT112 cells were not significantly different from those of the parental cell lines listed in table 1. Therefore, a direct comparison of responses to antitumor drugs and mechanisms associated with altered drug responses between parental and x-irradiation-treated cells was possible when identical cell culture conditions and drug exposure durations were used.

Drug Sensitivities of Parental and X-irradiation-treated Sublines

Responses of parental and x-irradiation-treated cells to a range of antitumor drugs were assessed by colony-forming assays. The L5178Y cells were cloned in 0.18% agar, as described earlier (*13*). The soft agar clonogenic assay method of Courtenay et al. (*14*) was used with the HN-1 and SuSa cell lines, whereas the MCF-7 and RT112 cell lines were cloned directly onto plastic (table 1). The IC_{50} after a 24-hour drug exposure have been reported previously for the two L5178Y cell lines (*6*) and for both paren-

---

ABBREVIATIONS: ID_{90} = dose of x-irradiation required to reduce cell survival by 90%; IC_{50} = concentration(s) of drug required to inhibit cell survival by 50% of untreated controls; VP-16-213 = etoposide; IC_{90} = concentration of drug required to reduce cell survival by 90% of untreated controls.

[1] Laboratory of Cellular Chemotherapy, Imperial Cancer Research Fund, London, England.

* *Reprint requests:* Bridget T. Hill, Ph.D., Laboratory of Cellular Chemotherapy, Imperial Cancer Research Fund, Lincoln's Inn Fields, P.O. Box 123, London WC2A 3PX, England.

Table 1.—Human tumor cell line characteristics

Cell line	Tumors of origin, reference	Population doubling time, hr[a]	Cell volume, μm^{3a}	Colony-forming efficiency, %	Reference
HN-1	Squamous cell carcinoma of the tongue	30±3	3,008±129	13[b]	(*7*)
MCF-7	Breast carcinoma	36±3	2,590±30	54[c]	(*8*)
RT112	Bladder carcinoma	26±2	1,669±22	10[c]	(*9*)
SuSa	Testicular teratoma	27±3	2,374±166	6[b]	(*10*)

[a] Values are the mean±SE.

[b] Cell lines were assayed in 0.3% agar.

[c] Cell lines were assayed on plastic.

tal and x-irradiated HN-1 cell lines (*11*). In both cases, the x-irradiation-treated sublines expressed resistance to vincristine and VP-16-213, unaltered responses to doxorubicin, and collateral sensitivity to cisplatin compared with their respective parental cells. In a more recent study (*12*), MCF-7/DXR_{10} cells were shown to express resistance to vincristine and VP-16-213 and unaltered responses to both doxorubicin and cisplatin compared with MCF-7 parental cells. A comparison of the drug sensitivities of parental and x-irradiation-treated SuSa and RT112 cells to these antitumor agents has now been made. Both x-irradiation-treated sublines express resistance to vincristine and VP-16-213. However, RT112/DXR_8 cells show collateral sensitivity to cisplatin, whereas SuSa/DXR_{10} cells express resistance to this agent. In addition, RT112/DXR_8 cells and SuSa/DXR_{10} cells exhibit an unaltered response to doxorubicin. These results are summarized in table 2, which shows a comparison of IC_{50} values of the x-irradiation-treated and parental cell lines exposed to vincristine, VP-16-213, doxorubicin, or cisplatin.

DERIVATION, CHARACTERIZATION, AND EVALUATION OF DRUG RESPONSES OF DRUG-TREATED SUBLINES

Derivation of Drug-treated Sublines

The establishment of a vincristine-resistant L5178Y cell line has been described (*15*). Drug-treated human tumor cell lines have been developed either by continuous exposure or pulsed 24-hour exposures of logarithmically growing parental cells to vincristine or VP-16-213. By these methodologies, MCF-7 cell lines treated with vincristine and HN-1 and SuSa cell lines treated with VP-16-213 have been developed. The cellular characteristics of each of these drug-treated cells were not significantly different from those of the parental cell lines listed in table 1. Therefore, we assessed drug responses of these cells by using identical culture conditions and drug exposure times. The IC_{50} values, derived from colony-forming assays after a 24-hour exposure to the selecting drug, for each of the parental and drug-resistant sublines are listed in table 3.

Table 2.—Summary of drug responses expressed by x-irradiation-treated or drug-treated human tumor cell lines compared with parental cell lines[a]

Cell lines	Vincristine	VP-16-213	Doxorubicin	Cisplatin
L5178Y/DXR_{10}	R+++[b]	R+++	NC[c]	CS+[d]
L5178Y/VCR_R	R+++	NC	R+++	NC
HN-1/DXR_{11}	R++	R++	NC	CS+
HN-1/VP_2	R++	R+++	R++	NC
MCF-7/DXR_{10}	R+++	R+++	NC	NC
MCF-7/VCR6E	R+++	R+++	R++	NC
SuSa/DXR_{10}	R++	R++	NC	R+++
SuSa/VP_{10}	R++	R+++	R+	NC
RT112/DXR_8	R+	R++	NC	CS+

[a] Data are presented as the ratio of IC_{50} values for a 24-hr drug exposure of each resistant subline compared with the parental line.

[b] R+++=resistant with a ratio of 3 to 10; R++=ratio of 1.8 to 2.9; R+=ratio of 1.5; underlining of R denotes selecting drug.

[c] NC=unchanged response, ratio of 0.9 to 1.3.

[d] CS+=collateral sensitivity with a ratio of 0.5 to 0.75.

Drug Sensitivities of Parental and Drug-treated Sublines

Antitumor drug responses of parental and drug-treated cell lines have been assessed by comparisons of IC_{50} values after a 24-hour exposure to drug (table 2). Vincristine-treated L5178Y cells express resistance to vincristine, cross-resistance to doxorubicin, and unaltered responses to cisplatin and VP-16-213. The HN-1 cells, which were exposed continuously to VP-16-213 (HN-1/VP2), express resistance to VP-16-213, cross-resistance to vincristine and doxorubicin, and an unchanged response to cisplatin. Similarly, MCF-7 cells, which were selected by six pulsed 24-hour exposures to vincristine (MCF-7/VCR6E), express resistance to vincristine and cross-resistance to VP-16-213 and doxorubicin. These cells also exhibit an unaltered response to cisplatin compared with parental cells. In addition, SuSa

Table 3.—Summary of orders of drug resistance resulting from continuous or pulsed exposure of parental cells to either vincristine or VP-16-213 as the selective agent[a]

	IC_{50} values, ng/ml[b]	
Cell lines	Vincristine	VP-16-213
L5178Y/P	0.28	—
L5178Y/VCR_R	1.20	—
HN-1/P	—	101
HN-1/VP_2	—	393
MCF-7/P	0.83	—
MCF-7/VCR6E	11.30	—
SuSa/P	—	7.5
SuSa/VP_{10}	—	35.0

[a] Details of drug exposures used for each human tumor cell line are provided in the text (section dealing with drug sensitivities of parental and drug-treated sublines).

[b] Values for 24-hr drug exposures were derived from full dose-response curves resulting from clonogenic cell survival assays.

cells, which were treated with six pulsed 24-hour exposures to VP-16-213 (SuSa/VP10), express significant resistance to VP-16-213 and cross-resistance to vincristine. They also show resistance to doxorubicin but an unaltered response to cisplatin.

Therefore, it is apparent that selection of resistant human tumor cell lines by exposure to drug has resulted in cells that generally express resistance to a variety of structurally unrelated antitumor agents, including VP-16-213, vincristine, and doxorubicin. This is in contrast to those drug-resistant cell lines derived by exposure to fractionated x-irradiation in which cross-resistance is not generally expressed to doxorubicin (although resistance to vincristine and VP-16-213 is consistently observed). In addition, considerable variations are noted in their responses to cisplatin, including in three instances the expression of collateral sensitivity (table 2).

Therefore, we found it worthwhile to compare possible mechanisms of resistance and collateral sensitivity in x-irradiation-treated and drug-treated cell lines, inasmuch as we have proposed that these may also differ depending upon the agent used to induce resistance.

INVESTIGATIONS OF MECHANISMS OF DRUG RESISTANCE AND COLLATERAL SENSITIVITY IN TUMOR CELL LINES DERIVED BY FRACTIONATED X-IRRADIATION PRETREATMENT OR BY DRUG EXPOSURE

Uptake of Tritiated Vincristine

Resistance to vincristine in vitro has been associated predominantly with reduced cellular accumulation of drug (*16*). Therefore, a comparison was made of uptake of [^{3}H]vincristine into parental cells and into the resistant sublines derived following x-ray or drug exposure. Initial data with the murine L5178Y cell lines, published earlier (*17*), showed that the vincristine-treated resistant cells had significantly reduced drug uptake and binding compared with the parental cells. In contrast, neither [^{3}H]vincristine influx or efflux were altered in the x-irradiation-treated subline. The results shown in figure 1 indicate that resistance to vincristine in HN-1/VP2 cells is also associated with a significantly ($P<.01$) reduced accumulation of drug compared with the HN-1 parental cells after both 30- or 60-minute exposures to 1 μM drug. This concentration of vincristine for a 1-hour exposure had no effect on cell survival. However, [^{3}H]vincristine uptake by the HN-1/DXR$_{11}$ cells was not modified.

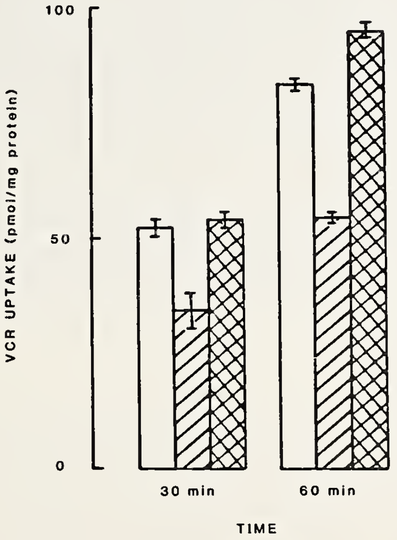


FIGURE 1.—Uptake of [^{3}H]vincristine (VCR) into HN-1 parental (*open square*), VP2 (*hatched*), and DXR$_{11}$ (*cross-hatched*) cells. Cells were cultured in glass petri dishes, and exponentially growing cells were exposed to [^{3}H]vincristine (1 μM) in medium containing serum for 30 or 60 min at 37° C. Medium containing [^{3}H]vincristine was removed, and cells were washed with ice-cold phosphate-buffered saline three times. Cells were then solubilized in 0.1 N sodium hydroxide (1 hr at 20° C), and aliquots were removed for scintillation counting and protein determination. Each experiment was the mean of two estimations. Histograms shown are the mean of 4 experiments ± SE.

These data indicate that selection of drug-resistant tumor cell lines by exposure either to vincristine (L5178Y/VCR$_R$) or VP-16-213 (HN-1/VP2) has resulted in the expression of resistance to vincristine that is associated with impaired drug uptake. Our findings also suggest that the development of drug-resistant tumor cell lines by exposure to fractionated x-irradiation has resulted in resistance to vincristine that is not associated with alterations in cellular uptake of drug.

The implication of these findings is that mechanisms of resistance to vincristine may differ depending upon the agent used to induce resistance.

Analyses of Etoposide-induced DNA Single-strand Breakage in HN-1 Cells

Both the x-irradiation and VP-16-213 pretreated HN-1 sublines expressed resistance to VP-16-213 that was not associated with any alterations in its cellular accumulation (*18*). Wozniak and Ross (*19*) have shown VP-16-213 to possess potent DNA damaging activity in the form of DNA single-strand breaks, double-strand breaks, and DNA–protein cross-links. In addition, resistance to VP-16-213 has been associated with a reduced formation of drug-induced DNA lesions in human lung and colon carcinoma cell lines (*20*), Chinese hamster ovary cells selected for resistance to VM-26 and VP-16-213 (*21*), and Chinese hamster lung cells selected for resistance to 9-hydroxyellipticine that expressed cross-resistance to VP-16-213 (*22*). Therefore, the extent of formation of VP-16-induced DNA strand breaks has been estimated in HN-1 cells. From table 4 it can be seen that

TABLE 4.—Comparison of cell survival and DNA single-strand breakage after exposure of exponentially growing HN-1 cells to VP-16-213[a]

Cell line	Cell survival, %	DNA single-strand breaks, rad equivalents
HN-1/P	16.1 ± 2.2	969 ± 112
HN-1/VP2	43.8 ± 3.9[b]	560 ± 84[b]
HN-1/DXR$_{11}$	28.2 ± 2.5[c]	380 ± 33[b]

[a] Cells were exposed to 10 μg VP-16-213/ml for 1 hr. The DNA single-strand breakage was estimated by the technique of alkaline elution, as described by Bedford et al. (*23*), and rad equivalent values were calculated by the fraction of sample cell DNA retained at 35% retention of an internal standard DNA relative to an irradiated control sample, as described by Kohn et al. (*24*). Values are ± SE.

[b] Difference was statistically significant from HN-1/P ($P<.01$).

[c] Difference was statistically significant from HN-1/P ($P<.05$).

both differently derived VP-16-213-resistant HN-1 cell lines sustain significantly fewer DNA single-strand breaks after drug exposure (10 μg/ml, 1 hr). However, significantly more single-strand breaks were induced in HN-1/VP2 cells than in the less resistant HN-1/DXR_{11} subline. The biochemical mechanisms responsible for reduced DNA single-strand breakage are at present unknown but may involve alterations in topoisomerase II activity (*21*).

Cisplatin-induced DNA Cross-linking in X-irradiation-treated RT112 Cells Expressing Collateral Sensitivity to Cisplatin

The RT112 cells which had been treated with fractionated x-irradiation (RT112/DXR_8) express collateral sensitivity to cisplatin compared with parental cells (table 2). The extent of cisplatin-induced DNA–DNA interstrand cross-links has been correlated with cytotoxicity in normal and transformed human fibroblasts (*25*) and in 2 human bladder tumor cell lines expressing differential sensitivities to the drug (*26*). Therefore, we decided to quantitate these lesions in RT112/DXR_8 and RT112 parental cells.

Peak values of cross-linking (rad equivalents) are shown in table 5 for RT112/P and RT112/DXR_8 cells 5 hours after a 1-hour exposure to equimolar (20 μg/ml) or equitoxic (IC_{90}) cisplatin concentrations. A significantly greater number of cross-links could be detected in the more sensitive RT112 (DXR_8) cell line in comparison to the parental cells. Conversely, after equitoxic concentrations of drug, the number of cross-links were similar in both lines. These results indicate that increased levels of DNA cross-linking may be a feature of the enhanced sensitivity to cisplatin of these x-ray-pretreated RT112 cells.

CONCLUSIONS

Drug-resistant human tumor cell lines can be derived in vitro by exposure either to fractionated x-irradiation or to antitumor drugs. However, patterns of cross-resistance and collateral sensitivity expressed by these differently derived sublines vary depending upon the agent used to induce resistance.

Derivation of drug-resistant human tumor cell lines by exposure to drug appears to select cells that express characteristics consistent with the multidrug-resistant phenotype (*27–30*), i.e., cross-resistance is expressed to a variety of structurally unrelated agents. However, the derivation of drug-resistant cell lines by fractionated x-irradiation treatment appears, in the tumor lines used in this study, to select cells that express resistance to vincristine and VP-16-213, but fail to show resistance to doxorubicin and vary in their responses to cisplatin.

Investigations into possible mechanisms associated with the altered drug responses expressed by these differently derived cell lines have indicated that resistance to vincristine expressed by cells selected by exposure to drug was associated with reduced accumulation of vincristine, which is consistent with the multidrug-resistant phenotype (*27–30*). Conversely, fractionated x-irradiation has resulted in vincristine-resistant cell lines with no impaired uptake of this drug. These results suggest that mechanisms of resistance to vincristine may differ, depending upon the agent used to induce resistance.

In contrast to this observation, our attempts to identify mechanisms of resistance to VP-16-213 in the HN-1 cells, in which resistance was selected by x-irradiation or drug exposure, have indicated that modified VP-16-213 transport was not implicated in either subline (*18*) and that both sublines sustained less DNA single-strand breakage than parental cells after VP-16-213 exposure. However, it would appear that the nature of the DNA strand breaks induced in these sublines (Lock RB, Hill BT, manuscript in preparation) may be different and might account for the observation that more single-strand breaks were induced in the more resistant subline (table 4).

Collateral sensitivity to cisplatin expressed by x-irradiated RT112 cells was associated with enhanced DNA cross-linking after exposure to this drug. Recently, we demonstrated a correlation between sensitivity to cisplatin and the extent of DNA cross-linking in 2 previously untreated human bladder carcinoma cell lines of independent origin (*26*). In these latter cell lines, enhanced cross-linking was accompanied by an increase in binding of the drug to DNA, and this was thought to be a consequence of lower glutathione levels in the more sensitive cell line (unpublished data). Whether a similar phenomenon is responsible for the enhanced sensitivity to cisplatin of the x-irradiation-pretreated RT112 subline compared with the parental line is currently under investigation. Nevertheless, this is one of the few reports whereby collateral sensitivity to a drug appears to be induced by prior exposure to x-irradiation (*6,11*).

Finally, these unique experimental systems appear to provide useful models for studying drug–radiation interactions with clinically relevant degrees of drug resistance and, if confirmed, our findings may have important clinical implications for the combined modality treatments of human cancers.

TABLE 5.—Comparison of DNA–DNA interstrand cross-linking in RT112/P and RT112/DXR_8 cells[a]

Cell line	DNA–DNA interstrand cross-links[b]	
	20 μg/ml	IC_{90}
RT112/P	58.2 ± 3.2	10.9 ± 0.9
RT112/DXR_8	87.8 ± 15.8	11.0 ± 2.0

[a] Cells were assessed 5 hr after a 1-hr exposure to equimolar (20 μg/ml) or equitoxic (IC_{90}) concentrations of cisplatin.

[b] Cross-linking was measured by alkaline elution as described by Kohn et al. (*24*). Values are expressed as rad equivalents ± SE.

REFERENCES

(*1*) PRICE LA, HILL BT: Safe and effective combination chemotherapy without *cis*-platinum for squamous cell carcinomas of the head and neck. Cancer Treat Rep 65(suppl 1):149–154, 1981.

(*2*) WOLF GT, CHRETIEN PB: Cancer of the head and neck. *In* The Chemotherapy and Immunotherapy of Head and Neck Cancer (Suen JY, Myers EN, eds). New York: Churchill Livingstone, 1981, pp 782–820.

(*3*) HOLLAND JF, GLIDEWELL O, COOPER RG: Adverse effect of radiotherapy on adjuvant chemotherapy of the breast. Surg Gynecol Obstet 150:817–821, 1980.

(*4*) YOUNG RC, KNAPP RC, PEREZ CA: Cancer of the ovary. *In* Cancer: Principles & Practice of Oncology (DeVita VT Jr, Hellman S, Rosenberg SA, eds). Philadelphia: Lippincott, 1982, pp 884–913.

(*5*) CAVALLI F, GOLDHIRSCH A, JOSS R: Single-agent activity and European experiences with etoposide in the treatment of small cell lung cancer. *In* Etoposide (VP-16): Current Status and New Developments (Issell BF, Muggia FM, Carter SK, eds). New York: Academic Press, 1984, pp 163–169.

(*6*) Bellamy AS, Hill BT: Murine L5178Y cells resulting in altered drug sensitivities from fractionated radiation exposure in vitro. JNCI 72:411–417, 1984.

(*7*) Easty DM, Easty GC, Carter RL, et al: Ten human carcinoma cell lines derived from squamous carcinomas of the head and neck. Br J Cancer 43:772–785, 1981.

(*8*) Soule HD, Vazquez J, Long A, et al: A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409–1413, 1973.

(*9*) Masters JRW, Hepburn PJ, Walker L, et al: Tissue culture model of transitional cell carcinoma: Characterization of twenty-two human urothelial cell lines. Cancer Res 46:3630–3636, 1986.

(*10*) Hogan B, Fellous M, Avner P: Isolation of a human teratoma cell line which expresses F9 antigen. Nature 270:515–518, 1977.

(*11*) Hill BT, Bellamy AS: Establishment of an etoposide-resistant human epithelial tumour cell line in vitro: Characterization of patterns of cross-resistance and drug sensitivities. Int J Cancer 33:599–608, 1984.

(*12*) Hill BT, Whelan RDH, Bellamy AS: Fractionated radiation (DXR) exposure in vitro results in altered drug responses in murine and human continuous tumor cell lines. Proc Am Assoc Cancer Res 25:331, 1984.

(*13*) Hill BT, Price LA, Goldie JH: The value of Adriamycin in overcoming resistance to methotrexate in tissue culture. Eur J Cancer 12:541–549, 1976.

(*14*) Courtenay VD, Selby PJ, Smith IE, et al: Growth of human tumour cell colonies from biopsies using two soft-agar techniques. Br J Cancer 38:77–81, 1978.

(*15*) Hill BT, Whelan RDH: Establishment of vincristine-resistant and vindesine-resistant lines of murine lymphoblasts in vitro and characterisation of their patterns of cross-resistance and drug sensitivities. Cancer Chemother Pharmacol 8:163–169, 1982.

(*16*) Beck WT: Cellular pharmacology of vinca alkaloid resistance and its circumvention. Adv Enzyme Regul 22: 207–227, 1984.

(*17*) Hill BT, Whelan RDH, Bellamy AS: Identification of differential mechanisms of resistance in vincristine-resistant cell lines developed either by exposure to drug or fractionated radiation. Cancer Treat Rev 11(suppl A): 73–79, 1984.

(*18*) Lock RB, Hill BT: In vitro drug resistance: Interactions between X-irradiation and etoposide. Br J Cancer 54:184, 1986.

(*19*) Wozniak AJ, Ross WE: DNA damage as a basis for 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-*β*-D-glucopyranoside) (etoposide) cytotoxicity. Cancer Res 43:120–124, 1983.

(*20*) Long BM, Musial ST, Newhouse LA, et al: Resistance to VP-16-213 and VM-26 is localized to nuclei of resistant human lung and colon carcinoma cell lines. Proc Am Assoc Cancer Res 26:348, 1985.

(*21*) Glisson B, Gupta R, Smallwood-Kentro S, et al: Characterization of acquired epipodophyllotoxin resistance in a Chinese hamster ovary cell line: Loss of drug-stimulated DNA cleavage activity. Cancer Res 46:1934–1938, 1986.

(*22*) Pommier Y, Schwartz RE, Zwelling LA, et al: Reduced formation of protein-associated DNA strand breaks in Chinese hamster cells resistant to topoisomerase II inhibitors. Cancer Res 46:611–616, 1986.

(*23*) Bedford P, Berger MR, Eisenbrand G, et al: The level of DNA interstrand crosslinking in bone marrow parallels the extent of myelosuppression in mice treated with four chloroethylnitrosoureas. J Cancer Res Clin Oncol 108: 141–147, 1984.

(*24*) Kohn KW, Ewig RAG, Erickson LC, et al: Measurement of strand breaks and cross-links by alkaline elution. *In* DNA Repair. A Laboratory Manual of Research Procedures (Friedberg EC, Hanawalt PC, eds), vol B. New York: Marcel Dekker, 1981, pp 379–401.

(*25*) Erickson LC, Zwelling LA, Ducore JM: Differential cytotoxicity and crosslinking in normal and transformed human fibroblasts treated with *cis*-diamminedichloroplatinum (II). Cancer Res 41:2791–2794, 1981.

(*26*) Bedford P, Masters JRW, Hill BT: Characterization of cisplatin-induced DNA damage in human bladder tumour cell lines. Biochem Soc Trans 14:617–618, 1986.

(*27*) Beck WT: Vinca alkaloid-resistant phenotype in cultured human leukemic lymphoblasts. Cancer Treat Rep 67: 875–882, 1983.

(*28*) Conter V, Beck WT: Acquisition of multiple drug resistance by CCRF-CEM cells selected for different degrees of resistance to vincristine. Cancer Treat Rep 68:831–839, 1984.

(*29*) Inaba M, Johnson RK: Uptake and retention of Adriamycin and daunorubicin by sensitive and anthracycline-resistant sublines of P388 leukemia. Biochem Pharmacol 27:2123–2130, 1978.

(*30*) Juliano RL, Ling V: A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta 455:152–162, 1976.

Cell-cycle, Phase-specific Cell Killing by Carmustine in Sensitive and Resistant Cells [1]

Peter A. Linfoot,[2] Mary Helen Barcellos-Hoff,[2] Thomas P. Brent,[3] Laurence J. Marton,[2] and Dennis F. Deen[2,4,*]

ABSTRACT—Cell-cycle, phase-specific cell kill caused by carmustine (BCNU) was measured in 4 cell lines with different sensitivities to the drug. Cells were treated with BCNU for 1 hour after which enriched subpopulations in various phases of the cell cycle were obtained by centrifugal elutriation and were assayed for cell survival. Levels of activity of guanine O^6-alkyltransferase were measured for each line; intracellular levels of this repair protein have been correlated with cellular resistance to chloroethylnitrosoureas. Only BTRC-19, a clone of the 9L line, had significant levels of alkyltransferase activity and exhibited a relatively flat age-response curve to BCNU. Alkyltransferase activity was not detected in the other 3 cell lines, all of which displayed a similar age response in which G_1- and G_2/M-phase cells were relatively sensitive to BCNU compared with the response of S-phase cells. We conclude that alkyltransferase activity may overwhelm other determinants that cause cell-cycle phase specificity to BCNU.—NCI Monogr 6:183–186, 1988.

The cytotoxicity of many cancer chemotherapeutic agents is cell cycle, phase specific (*1*), a property that can be important in combination treatment protocols (*2*). Clinical combination chemotherapeutic protocols designed to take advantage of the phase specificity of different drugs, however, have had limited success (*3*). This clinical failure may result from a lack of knowledge of factors that cause phase specificity and our inability to predict with accuracy how drugs will interact when they are given in combination.

A chloroethylnitrosourea with well-established activity against solid tumors, BCNU is thought to kill cells in a two-step process, first by formation of 2-chloroethyl monoadducts on DNA followed by a reaction with DNA nucleophiles to form both interstrand and intrastrand cross-links, the former of which are thought to be the lesion that leads to cell death (*4*). In 9L (*5,6*) and other cells (*7*), BCNU is less cytotoxic to cells in S phase at the time of treatment than to cells in G_1 and G_2/M phases. The reason for this phase specificity is uncertain.

We performed the experiments reported here to determine whether the phase specificity of BCNU was related either to the inherent sensitivity of cells to the drug or to the level of AT in the cells. There is a strong correlation between intracellular levels of AT, a repair protein that excises alkylation products on O^6-guanine and resistance to chloroethylnitrosoureas (*8*). Two 9L rat brain tumor and 2 CHO cell lines were chosen for our study. Cell survival after BCNU treatment was measured by determination of colony-forming ability of elutriated fractions enriched in the various phases of the cell cycle. The pattern of phase-specific cell kill reported for BCNU in 9L cells was confirmed and was found to be the same for the 2 CHO lines. However, the pattern was significantly different for BTRC-19 cells, which showed a relatively flat, highly resistant response to BCNU in all phases of the cell cycle. The 9L and the 2 CHO cell lines had no detectable levels of AT activity, whereas BTRC-19 had high levels of the repair protein.

MATERIALS AND METHODS

Cells and culture conditions.—The 9L rat brain tumor cell line is well established. The very resistant clone designated BTRC-19 was isolated from 9L cell spheroids treated with 45 μM BCNU (Barcellos-Hoff MH, Deen DF, unpublished results). The AA8 are wild-type CHO cells; the cell line designated UV-4 was isolated from AA8 cells that were treated with ethylnitrosourea and is sensitive to UV light and DNA cross-linking agents (*9*).

Monolayer cultures of the 4 lines were maintained at 37° C in a 5% CO_2–95% air environment. Both 9L and BTRC-19 cells were cultured in CMEM consisting of Eagle's MEM supplemented with nonessential amino acids, 50 μg gentamycin/ml, and 10% newborn calf serum. The UV-4 and AA8 cells were cultured in alpha-MEM without nucleosides and supplemented with 10% fetal calf serum and 50 μg gentamycin/ml.

After 9L and BTRC-19 cells were removed from 75-cm^2 plastic flasks by a rinsing with 3 ml saline A containing 0.05% trypsin and 0.02% versene, the rinse was decanted, another 3 ml of this saline was added, and flasks were incubated at 37° C for 5 minutes. Enzyme action was

---

ABBREVIATIONS: BCNU = carmustine; AT = guanine-O^6-alkyltransferase; CHO = Chinese hamster ovary; CMEM = complete minimum essential medium; PE = plating efficiency (efficiencies).

---

[1] Supported by Public Health Service grants CA-13525, CA-42779, and CA-36888 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and the Andres Soriano Cancer Research Fund.

[2] Brain Tumor Research Center, Department of Neurological Surgery (*P. A. Linfoot, M. H. Barcellos-Hoff, L. J. Marton,* and *D. F. Deen*), Department of Radiation Oncology (*D. F. Deen*), and Department of Laboratory Medicine (*L. J. Marton*), School of Medicine, University of California, San Francisco.

[3] Department of Clinical and Experimental Pharmacology, St. Jude Children's Research Hospital, Memphis, TN.

[4] We thank Dr. Larry Thompson for providing the CHO cells, Frances James for typing the manuscript in draft, and Neil Buckley for editing it.

* *Reprint requests:* Dennis F. Deen, Ph.D., Brain Tumor Research Center (783-HSW), Department of Neurological Surgery, University of California, San Francisco, San Francisco, CA 94143-0520.

stopped by the addition of 10 ml CMEM; the flask was struck sharply, and the cell suspension was pipetted several times so we could obtain single cells. The 9L and BTRC-19 cells grown in 850-cm^2 roller bottles were removed by a rinsing with 50 ml of Hanks' balanced salt solution, after which an enzyme cocktail (0.05% pronase, 45 Kaken proteolytic units/ml; 0.02% DNAse, 7×10^4 dornase units/mg; and 0.02% collagenase II, 139 units/mg) was added for 30 minutes at 37° C; the resulting suspension was diluted with ice-cold CMEM and centrifuged. We resuspended the pellet in fresh CMEM and passed it through a 25-μ nylon mesh screen to obtain a single cell suspension. The AA8 and UV-4 cells grown in either 75-cm^2 plastic flasks or 850-cm^2 roller bottles were disaggregated with the use of the saline A–trypsin–versene procedure.

Drug treatment.—The BCNU was stored at −70° C. Immediately before use, it was dissolved in a small amount of 100% ethanol and then diluted in CMEM in treatment flasks. The concentration of ethanol was always less than 1% and did not affect PE. Extracellular pH was maintained between 7.2 and 7.4 at all times; drug and cells were incubated at 37° C. After drug treatment, cultures were rinsed with fresh CMEM and processed for cell survival. Because phase-specific BCNU cell kill is a dose-modified effect (*6*), drug concentrations that cause a 1–2 log cell kill were chosen for treatment of the 4 cell lines.

Cell survival assay.—The colony-forming efficiency assay we used to measure 9L and BTRC-19 cell survival has been described (*9*). Briefly, cells were counted, diluted, and plated (in quadruplicate) into petri dishes containing 5×10^4 irradiated (40 Gy) autologous feeder cells. After incubation for 12 to 14 days, colonies were fixed with ethanol, stained with crystal violet, and counted. The PE was calculated as the ratio of the number of colonies counted to the number of cells plated times 100. Surviving fraction was calculated as the PE of treated cells divided by the PE of untreated cells.

Survival of CHO cells was also measured with a colony-forming efficiency assay (*10*), performed essentially as described above with the exceptions that no feeder cells were used and colonies were incubated for 7 to 9 days.

Centrifugal elutriation.—Subpopulations of BCNU-treated cells enriched in the various phases of the cell cycle were obtained by centrifugal elutriation with a Beckman J2-21 centrifuge equipped with a JE-6 elutriator rotor as described (*5,6*). In brief, after drug treatment, cultures were disaggregated to single cells, placed in the elutriator flow system, and allowed to equilibrate in the separation chamber before elutriation. The rotor speed and fluid flow rate were determined empirically for each cell line, and cells enriched in G_1, S, and G_2/M phases were obtained by reduction of the rotor speed in 100-rpm decrements; at each rotor speed setting, 270-ml fractions were collected. After elutriation, cells were centrifuged at 1,000 rpm for 15 minutes, resuspended in ice-cold CMEM, and held on ice until they could be assayed for cell survival. The cell number and median cell volume of each elutriator fraction were monitored on a Coulter counter equipped with a channelyzer.

The enrichment of each fraction was determined by analysis of single parameter DNA histograms obtained by flow cytometry (*6*). Cells were fixed with 70% ethanol, stained with chromomycin A_3 solution, and samples were analyzed with an FACS III flow cytometer with a 5-watt argon laser set at 457 nm and adjusted to emit 160–200 milliwatts. The resulting fluorescence was passed through a Schott KV-250-nm, long-wave pass filter. The computer program PEAKS3 at Lawrence Livermore National Laboratory was used for the analysis of DNA distributions.

Guanine O^6*-alkyltransferase assay.*—The transfer of tritium-labeled methyl groups from O^6-guanine in DNA to the AT acceptor protein was determined from the appearance of tritium in an insoluble protein fraction isolated from BCNU-treated cells by essentially the method described by Myrnes et al. (*11*). About 1 μg tritium-labeled *N*-methyl-*N*-nitrosourea-treated DNA was incubated with the transferase at 37° C for 30 minutes in 200 μl buffer containing 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, 1 m*M* dithiothreitol, 0.02% sodium azide, and 10% glycerol. The reaction was stopped by the addition of 2.5 volumes of 5% trichloroacetic acid to precipitate DNA and protein. The DNA was selectively hydrolyzed by heating for 30 minutes at 80° C, which removes all methylpurines, then cooled on ice for at least 5 minutes, and when less than 100 μg protein was present [protein assayed according to Lowry et al. (*12*)], 100 μg bovine serum albumin was added. The protein precipitate was collected by filtration on 2.5-cm Whatman GF-F glass fiber filters. The filters were washed twice with 15 ml 5% trichloroacetic acid and once with 95% ethanol. Each filter was placed in 200 μl of solubilizer (NCS) in a scintillation vial for about 1 minute before the addition of a toluene-based nonaqueous scintillation cocktail for counting. A unit of transferase activity was equivalent to 1 pmol tritium-labeled methyl bound to protein in this assay.

RESULTS

The BCNU dose–response curves for asynchronous cultures of the 4 lines studied are shown in figure 1. The dose scales are different in the 2 figures; if plotted on the same scale, curves for 9L (fig. 1A) and AA8 (fig. 1B) would be similar.

The phase-specific survival patterns for the parent 9L and BTRC-19 are illustrated in figure 2. In contrast to the parent line, BTRC-19 cells showed little variation in phase-specific survival even at a dose of 250 μM BCNU, which reduced the PE of asynchronous cells to about 3%.

In the phase-specific survival patterns for AA8 cells and UV-4 cells (fig. 3), the age response for both lines is essentially the same as that obtained for 9L cells, with S-phase cells less sensitive than G_1- or G_2/M-phase cells. That S-phase UV-4 cells were relatively resistant to BCNU indicates that the biochemical mechanism responsible for the increased sensitivity to cross-linking agents does not affect BCNU phase specificity.

No AT activity was found in 9L, AA8, or UV-4 cells, but BTRC-19 cells had high levels (2.24 pmol activity/mg protein) of AT. This level of activity is comparable to that seen in the human lymphoblast cells used as a positive control in the assay (*13*).

DISCUSSION

The S-phase cells elutriated from cultures of UV-4, AA8, and 9L cells treated with BCNU at doses that cause a 1–2 log cell kill in asynchronous cells were more resistant than cells in other phases of the cell cycle. In contrast, little variation in cell kill was noted throughout the cell cycle for

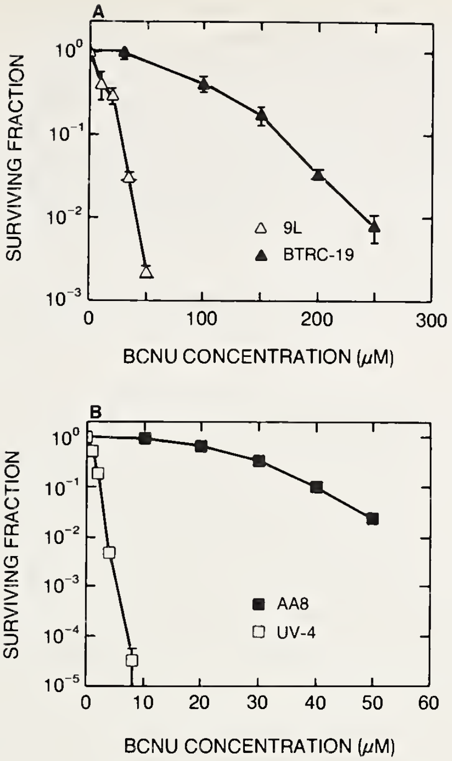


FIGURE 1.—Survival curves for 9L and BTRC-19 cells (A), and AA8 and UV-4 cells (B) treated with BCNU for 1 hr. Each point represents the mean ± SD of 4–8 petri dishes. The PE for untreated cells were 9L, 69.5 ± 12.4; BTRC-19, 37.3 ± 6.7; AA8, 76.7 ± 6.5; and UV-4, 73.2 ± 11.7.

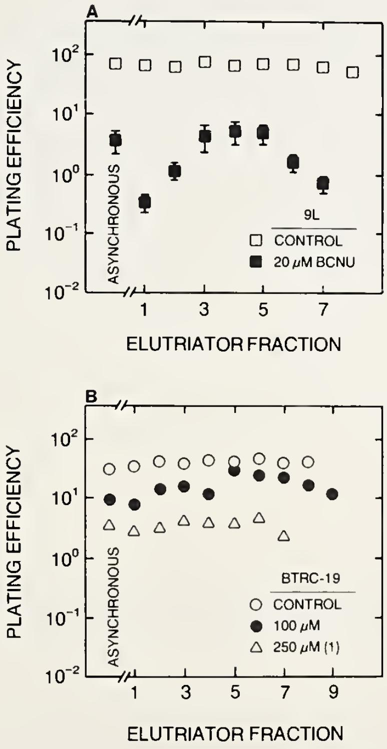


FIGURE 2.—The PE of 9L (A) and BTRC-19 (B) cells treated with BCNU for 1 hr. Control represents untreated elutriated cells. Each point represents the mean ± SD of 4–8 petri dishes.

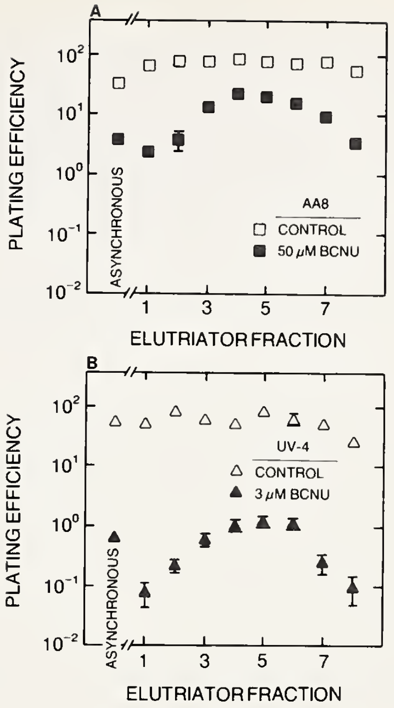


FIGURE 3.—The PE of AA8 (A) and UV-4 (B) cells treated with BCNU for 1 hr. Control represents untreated elutriated cells. Each point represents the mean ± SD of 4–8 petri dishes.

BTRC-19 cells treated at doses that produced a similar asynchronous survival level. Only BTRC-19 cells had significant levels of AT activity. Thus the lack of AT activity and the similar age response of 9L, AA8, and UV-4 cells indicates that AT-mediated repair is not involved in the phase specificity in these cells.

The reason for the phase specificity of BCNU in 9L, AA8, and UV-4 cells remains to be determined, but it is unlikely that phase-specific cell killing is related to doubling times or phase durations of the cells. The cell cycle time of exponentially growing 9L cells is approximately 20 hours, and the durations of G_1, S, and G_2 phases are 8, 8, and 3.5 hours, respectively; mitosis requires about 0.5 hour (*14*). The doubling time of BTRC-19 cells is similar to 9L cells, although the former cells have a slightly longer lag phase that may be related to their lower PE (fig. 1). Cell-cycle phase durations have not been determined in BTRC-19 cells, but because the percentages of G_1-, S-, and G_2/M-phase cells during exponential growth (data not shown) and the doubling times are similar to 9L cells, we assume that the phase durations are similar. Both AA8 and UV-4 cells have doubling times of 13–14 hours (*10*) and similar PE (fig. 1). Although the cell-cycle phase durations have not been determined in these cell lines, reported durations of G_1, S, and G_2/M for CHO cells are 2–4, 5–7, and 2–3 hours, respectively (*15,16*). The finding that 9L, AA8, and UV-4 cells have similar patterns of phase specificity to BCNU treatment suggests that differences in cell-cycle parameters are not important for the observed age response.

We (unpublished results) recently found that, compared with exponentially growing 9L cells (*17*), the increased BCNU sensitivity of cells in 9L multicellular spheroids is the result of a greater sensitivity of the noncycling cell population. Moreover, these cells are able to repair some BCNU damage if they are left noncycling in intact spheroids for 24 hours after treatment. Because noncycling cells rapidly enter the cell cycle when placed in monolayer culture, and because repair is not evident in exponentially growing cells in monolayer culture, damage may become fixed when BCNU-treated cells progress through the cell cycle. Thus the cell-cycle phase specificity toward BCNU may reflect the time available for repair before damage is fixed, a process similar to that seen for repair of x-ray damage (*18*). More studies are needed, however, if researchers are to clarify the role of DNA repair in phase-specific cell killing by chloroethylnitrosoureas.

Our results do indicate that in the absence of AT, other factors that influence the phase-specific survival become apparent. If the results reported here can be generalized to other cell lines and to cells in vivo, they may have important clinical implications. The majority of nontransformed human cell lines examined to date are of the MER^+ phenotype (*19*). Thus BCNU would not be expected to show phase-specific cell killing in these cells. Nonetheless, for cells of the MER^- phenotype, which are sensitive to BCNU, cell-cycle specificity would be important, particularly in clinical protocols with other anticancer agents.

REFERENCES

(*1*) Hill BT: Cancer chemotherapy. The relevance of certain concepts of cell cycle kinetics. Biochim Biophys Acta 516:389–417, 1978.

(*2*) Norton L: Implications of kinetic heterogeneity in clinical oncology. Semin Oncol 12:231–239, 1985.

(*3*) Gray JW: Quantitative cytokinetics: Cellular response to cell cycle specific agents. Pharmacol Ther 22:163–197, 1983.

(*4*) Weinkam RJ, Lin H-S: Chloroethylnitrosourea cancer chemotherapeutic agents. Adv Pharmacol Chemother 19:1–33, 1982.

(*5*) Bjerkvig R, Oredsson SM, Marton LJ, et al: Cell cycle age response of cells to 1,3-*bis*(2-chloroethyl-1-nitrosourea) and modification by α-difluoromethylornithine. Cancer Res 43:1497–1500, 1983.

(*6*) Linfoot PA, Gray JW, Dean PN, et al: Effect of cell cycle position on the survival of 9L cells treated with nitrosoureas that alkylate, cross-link, and carbamoylate. Cancer Res 46:2402–2406, 1986.

(*7*) Meyn RE, Murray D: Cell cycle effects of alkylating agents. Pharmacol Ther 24:147–163, 1984.

(*8*) Scudiero DA, Meyer SA, Clatterbuck BE, et al: Sensitivity of human cell strains having different abilities to repair O^6-methylguanine in DNA to inactivation by alkylating agents including chloroethylnitrosoureas. Cancer Res 44:2467–2474, 1984.

(*9*) Wheeler KT, Deen DF, Wilson CB, et al: BCNU modification of the in vitro radiation response of 9L rat brain tumor cells of rats. Int J Radiat Oncol Biol Phys 2:79–88, 1977.

(*10*) Thompson LH, Busch DB, Brookman K, et al: Genetic diversity of UV-sensitive mutants of Chinese hamster ovary cells. Proc Natl Acad Sci USA 78:3734–3737, 1981.

(*11*) Myrnes B, Norstrand K, Giercksky K-E, et al: A simplified assay for O^6-methylguanine–DNA and methyltransferase activity and its application to human neoplastic and non-neoplastic tissues. Carcinogenesis 5:1061–1064, 1984.

(*12*) Lowry OH, Rosebrough NJ, Farr AL, et al: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275, 1951.

(*13*) Brent TP: Inactivation of purified human O^6-alkylguanine–DNA alkyltransferase by alkylating agents or alkylated DNA. Cancer Res 46:2320–2323, 1986.

(*14*) Nomura K, Hoshino T, Deen DF, et al: Perturbed cell kinetics of 9L rat brain tumor cells following dianhydrogalactitol. Cancer Treat Rep 62:2055–2061, 1978.

(*15*) Rao AP, Rao PN: The cause of G_2-arrest in Chinese hamster ovary cells treated with anticancer drugs. J Natl Cancer Inst 57:1139–1143, 1976.

(*16*) Gray JW, Bogart E, Gavel DT, et al: Rapid cell cycle analysis. 11. Phase durations and dispersions from computer analysis of RC curves. Cell Tissue Kinet 16: 457–471, 1983.

(*17*) Deen DF, Hoshino T, Williams ME, et al: Development of a 9L rat brain tumor cell multicellular spheroid system and its response to 1,3-bis(2-chloroethyl)-1-nitrosourea and radiation. JNCI 64:1373–1382, 1980.

(*18*) Iliakis G, Nüsse M: Evidence that repair and expression of potentially lethal damage cause the variations in cell survival after X irradiation observed through the cell cycle in Ehrlich ascites tumor cells. Radiat Res 95:87–107, 1983.

(*19*) Yarosh DB: The role of O^6-methylguanine–DNA methyltransferase in cell survival, mutagenesis, and carcinogenesis. Mutat Res 145:1–16, 1985.

Chinese Hamster Pleiotropic Multidrug-resistant Cells Are Not Radioresistant

James B. Mitchell,* Janet Gamson, Angelo Russo, Norman Friedman, William DeGraff, James Carmichael, and Eli Glatstein[1]

ABSTRACT—The inherent cellular radiosensitivity of a Chinese hamster ovary pleiotropic cell line that is multidrug resistant (CH^RC5) was compared to that of its parental cell line (AuxB1). Radiation survival curve parameters n and D_0 were 4.5 and 1.1 Gy, respectively, for the CH^RC5 line and 5.0 and 1.2 Gy, respectively, for the parental line. Thus, the inherent radiosensitivity of the two lines was similar even though key intracellular free radical scavenging and detoxifying systems employing glutathione, glutathione transferase, and catalase produced enzyme levels that were 2.0-, 1.9-, and 1.9-fold higher, respectively, in the drug-resistant cell line. Glutathione depletion by buthionine sulfoximine resulted in the same extent of aerobic radiosensitization in both lines (~10%). Incorporation of iododeoxyuridine into cellular DNA sensitized both cell lines to radiation. These studies indicate that pleiotropic drug resistance does not necessarily confer radiation resistance.—NCI Monogr 6:187–191, 1988.

Over the past few years, several in vitro models demonstrating marked drug resistance have been developed. These systems allow the evaluation of inherent cellular sensitivity to a variety of agents including radiation. Bech-Hansen et al. (*1*), Ling (*2*), and Ling et al. (*3*) have developed and extensively studied such a series of Chinese hamster ovary cells exhibiting multidrug resistance. The pleiotropic drug resistance patterns found in the mutant cells developed by these investigators correlate with the expression of P-glycoprotein in the cell membrane and of altered drug permeability (*4–6*). The emergence of multidrug resistance in patients following chemotherapy poses a significant problem in further cancer treatment since these tumors become resistant to a variety of previously unused chemotherapeutic agents. Oncology protocols frequently employ chemotherapy and radiation therapy or radiation therapy alone to treat chemotherapy failures; therefore, it becomes important to determine if development of multidrug resistance also induces relative radiation resistance.

Several chemotherapeutic drugs as well as radiation have cytotoxic effects mediated through damage to DNA. The common thread that potentially unites several commonly used drugs and ionizing radiation is that toxic free radicals may interact lethally to alter the structure of DNA. The present study attempts to establish a basic radiobiologic framework concerning pleiotropic drug-resistant cells by evaluating the inherent cellular radiosensitivity of one of these multidrug-resistant cell lines. Furthermore, we present attempts 1) to find a correlation between the inherent radiosensitivity of these cell lines and intracellular levels of cellular redox-related biomolecules (GSH and cellular detoxifying enzymes with the potential to eliminate toxic free radicals) and 2) to determine the feasibility of using a radiosensitizing agent (IdUrd) to sensitize the multidrug-resistant cell to radiation.

MATERIALS AND METHODS

Cell culture.—Two Chinese hamster ovary cell lines supplied by Dr. V. Ling were used. The parental line AB was exposed to the mutagen ethyl methanesulfonate, and the surviving cells were grown for an extended period of time in increasing concentrations of colchicine. A clone, C5, which was resistant to colchicine and a variety of other chemotherapeutic agents, was ultimately identified (*3*). Both cell lines were cultured in F12 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. The doubling times of AB and C5 lines were 12 and 14.5 hours, respectively, with plating efficiencies of both lines ranging from 50%–80%. Forty-eight hours prior to experimental studies, cells were trypsinized from stock flasks, rinsed, plated in 100-mm petri dishes (3×10^5 cells per dish), and incubated at 37° C. Following various experimental protocols, cells were trypsinized, rinsed, counted, and plated for macroscopic colony formation. Dependent on the anticipated survival level, 10^2 to 10^5 cells were plated per dose point. When high cell numbers were plated, larger petri dishes were used to avoid possible cell-density effects. Following 8–10 days of incubation, colonies were fixed with methanol:acetic acid (3:1), stained with crystal violet, and counted; only colonies >50 cells were scored. All experiments were conducted at least twice. Survival curves were drawn through the data points by eye.

Drug exposure.—Daunomycin (daunorubicin) and melphalan were obtained from the National Cancer Institute (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment). The BSO was obtained from Chemalog (Chemical Dynamics Corp., South Plainfield, NJ), and the IdUrd was

---

ABBREVIATIONS: GSH = glutathione; IdUrd = iododeoxyuridine; AB = AuxB1 cell line; C5 = CH^RC5 cell line; BSO = buthionine sulfoximine; PBS = phosphate-buffered saline; SOD = superoxide dismutase.

[1] Radiation Oncology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

* *Reprint requests:* James B. Mitchell, Ph.D., Radiation Oncology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bldg. 10, Rm. B3B69, National Institutes of Health, Bethesda, MD 20892.

obtained from Sigma Chemical Co. (St. Louis, MO). For studies with daunomycin or melphalan, the medium was aspirated from the dishes and replaced with full medium containing various concentrations of the drugs. Following a 1-hour exposure at 37° C, the medium was removed, and the cell monolayer was rinsed twice with PBS and subsequently plated as described. The BSO pretreatment consisted of changing the medium 15 hours before x-ray exposure to medium containing 10 m*M* BSO. Following x-ray exposure, the cell monolayer was rinsed twice with PBS and plated as described. Pretreatment with IdUrd consisted of changing the medium 25 hours prior to x-ray exposure to medium containing 10^{-5} *M* IdUrd; following x-ray exposure, cells were plated as described.

X-ray exposure.—Cells were irradiated using a Varian Clinac 20 linear accelerator at a dose rate of 5 Gy/minute of 15-MeV x-rays. Full electron equilibrium was ensured for all exposures.

Glutathione and enzyme analysis.—Exponentially growing cultures of both cell lines were assayed for GSH and related detoxification enzymes. Cells were collected for GSH determinations, rinsed with cold PBS, and immediately treated with cold 0.6% sulfosalicylic acid. Total GSH content (reduced + oxidized) was assayed by the glutathione reductase procedure of Tietze (*7*). Enzyme assays were conducted after collecting the cells, rinsing with PBS, and resuspension in a known volume of PBS. Cells were lysed by freezing the cell suspension (−70° C) and thawing to room temperature twice. Aliquots were then taken for the various enzyme assays and protein determinations. Glutathione reductase was assayed according to the method of Massey and Williams (*8*), and the change in optical density at 340 nm (NADPH) per minute was determined. Glutathione peroxidase was assayed according to the method of Paglia and Valentine (*9*), in which the glutathione reductase/NADPH system is coupled to glutathione disulfide production via glutathione peroxidase.

Glutathione transferase activity was assayed by the method of Habig et al. (*10*). The reaction of GSH with the substrate 1-chloro-2,4-dinitrobenzene was monitored at 340 nm. The SOD activity was measured according to the assay of Marklund and Marklund (*11*). This assay is based on the ability of SOD to inhibit the autoxidation of pyrogallol. Catalase activity was determined by the method of Bergmeyer (*12*), observing the change in optical density at 240 nm as a function of time. Protein determinations were conducted according to the method of Bradford (*13*).

RESULTS

The C5 cell line has been shown to be resistant to a number of chemotherapy drugs including daunomycin and melphalan (*3*). Examples demonstrating this drug resistance are shown in figure 1. In this study, drug-resistant C5 cells were found to be 30-fold more resistant to daunomycin and sevenfold more resistant to melphalan than the parental cell line.

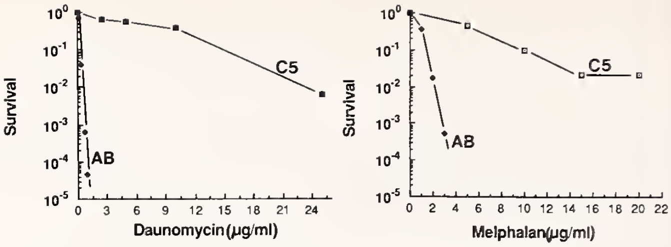


FIGURE 1.—Survival curves of AB and C5 cells exposed to daunomycin (*left*) and melphalan (*right*) for 1 hr. Relative resistances of C5 line (drug concentration ratio at 2% survival) for daunomycin and melphalan were 30 and 7, respectively.

Previously, some cells with induced drug resistance have been reported to have increased levels of GSH (*14–16*). To further characterize the cell lines in the present study, levels of GSH as well as other key detoxification enzymes were determined (table 1). In the drug-resistant C5 cell line, GSH, glutathione transferase, and catalase were elevated by factors of 2.0, 1.9, and 1.9, respectively, compared to the parental line. Levels of glutathione reductase and glutathione peroxidase were not different in the two cell lines, but SOD levels were higher in the AB than in the C5 cell line. Gamma-glutamyl transpeptidase, an enzyme involved in the transport of GSH and amino acids, was not detectable in either cell line (data not shown).

Radiation survival curves for the two cell lines are shown in figure 2. Individual survival points represent the mean ± SEM from six independent experiments. The radiation survival curve parameters *n* and D_0 were 4.5 and 1.1 Gy, respectively, for the C5 cell line and 5.0 and 1.2 Gy, respectively, for the AB line. Figure 3 shows the results of pretreating both cell lines with BSO prior to x-ray exposure. Exposure to BSO inhibits γ-glutamylcysteine synthetase (*17*) and, with time, results in GSH depletion (*18–22*). This BSO pretreatment did not alter the plating efficiency, growth rate, or cell-cycle distribution of either cell line (data not shown). However, after the 15-hour BSO treatment, GSH levels for both cell lines were <5% of control levels. The curves for BSO pretreatment show that BSO slightly sensitized both cell lines, compared to the respective control curves. Enhancement ratio was defined as the ratio of radiation doses for control and BSO-treated cells at 1% survival. The ratios were approximately 1.09 for both cell lines.

The effects of IdUrd incorporation on radiosensitivity of the AB and C5 cells are shown in figure 4. The enhancement ratios of IdUrd treatment for AB and C5 cells were 1.9 and 1.6, respectively. It should be noted that the lower enhancement ratio for the C5 cells compared to that for the AB cells is probably caused by the longer doubling time of C5 cells, resulting in less incorporation of IdUrd.

TABLE 1.—Levels of GSH and related detoxification enzymes in AB vs. C5 cells[a]

Enzyme	AB	C5
GSH (nmol/mg of protein)	13.0 ± 5.7	26.0 ± 5.2
GSH reductase[b]	13.9 ± 0.4	13.0 ± 4.0
GSH peroxidase[b]	7.7 ± 1.7	6.2 ± 0.9
GSH transferase[c]	49.2 ± 6.0	92.5 ± 26.0
SOD (μg/mg of protein)	3.7 ± 0.3	2.5 ± 0.4
Catalase (Bergmeyer units/mg of protein)	31.8 ± 3.5	60.0 ± 1.8

[a] Values are mean ± SEM; $n \geq 2$. Specific activity = units/mg of protein.

[b] 1 unit = 1 nmol of NADPH oxidized/min.

[c] 1 unit = 1 nmol of (1-chloro-2,4-dinitrobenzene reacted)/min.

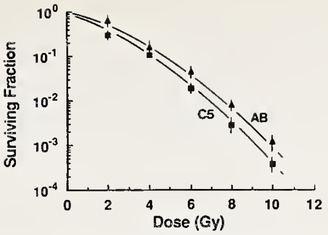


FIGURE 2.—X-ray survival curves for AB (▲) and C5 (■) cells. Results shown represent mean ± SEM of 6 individual experiments.

DISCUSSION

No cross-resistance of the multidrug-resistant cell C5 to ionizing radiation was observed. Inherent radiosensitivity was similar for the parental and the drug-resistant cell lines. Since radiation exposure results in the production of free radicals and toxic oxygen-related species, various detoxifying systems were evaluated in both cell lines. The drug-resistant cells were found to have higher levels of GSH, glutathione transferase, and catalase than the parental line. However, the radiosensitivity of these cell lines could not be correlated with levels of GSH or cellular detoxification enzymes. The lack of correlation between GSH levels and radiation sensitivity is consistent with previous studies (*23,24*). We have shown that when GSH levels were elevated approximately twofold in Chinese hamster cells by pretreatment with oxothiazolidine-4-carboxylate (*25*), no radioprotection (*23,24*) was afforded.

Recently, Wallner and Li (*26*) reported a lack of correlation between radiosensitivity and daunomycin resistance in a Chinese hamster cell line made resistant to daunomycin by long-term passage in increasing levels of the drug. Similar results have been obtained for daunomycin-resistant human breast tumor (Adr^{R}MCF-7) cells (Cowan K, personal communication). In contrast, Louie et al. (*16*) reported increased resistance to radiation in human ovarian cancer cells selected for melphalan and cisplatin resistance. These ovarian cell lines, which exhibited a modest change in D_0 compared to the parental cell lines, were also elevated in cellular GSH. Treatment with BSO for 48 hours (GSH = 15% of control) restored the radiosensitivity of one of these cell lines to parental values. However, with prolonged exposure time to BSO, it is possible that cell-cycle redistribution could have occurred, rendering the overall population more sensitive to radiation. It has long been known that cells vary in radiosensitivity as a function of their position in the cell cycle (*27*). Aerobic radiosensitization by BSO treatment has been reported by Mitchell et al. (*19*); GSH levels were rapidly depleted (GSH <5%) to avoid cell-cycle perturbations. Under these conditions, they reported approximately 10% radiosensitization by BSO treatment. These results relating GSH depletion to the extent of sensitization are similar to those observed in both cell lines in the present study. In general, it has been observed that extensive GSH depletion is necessary to sensitize cells to aerobic irradiation (*28*). Thus, the enhancement in radiosensitivity observed by Louie et al. (*16*), which was near 50% at the 1% survival level for an 85% reduction in GSH levels, could be explained by factors other than GSH levels. For example, it is not clear why these investigators incubated the cells in BSO medium 24 hours after irradiation (*16*). Additionally, if quantitative GSH levels were significant in conferring radiosensitivity, the two human cell lines derived from drug-refractory patients [OVCAR-$4^{(Ag+)}$ and OVCAR-$3^{nu(Ag+)}$] should have been markedly more radioresistant than the drug-sensitive human ovarian line (2780) reported by Louie et al. (*16*). The GSH levels in these cells were sixfold to eightfold higher than those in the drug-sensitive line; yet the radiosensitivity (based on *n*) of the three lines was similar (*16*).

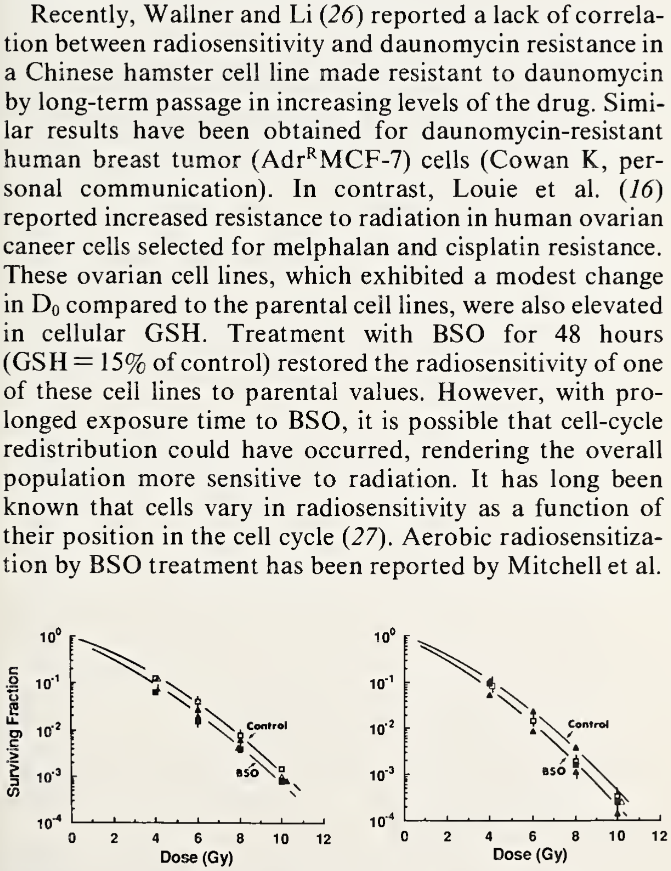


FIGURE 3.—X-ray survival curves for AB (*left*) and C5 (*right*) cells pretreated for 15 hr with 10 m*M* BSO. GSH levels for BSO pretreatment were <5% of control values at time of irradiation for both cell lines.

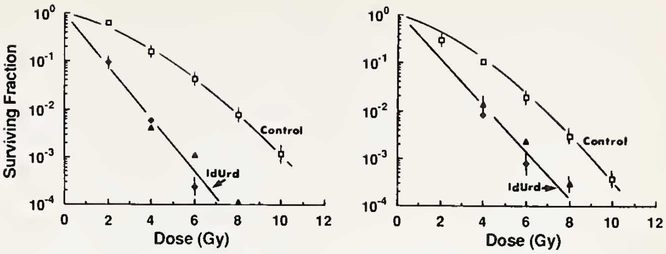


FIGURE 4.—X-ray survival curves of AB (*left*) and C5 (*right*) cells pretreated for 25 hr with 10 μ*M* IdUrd. Enhancement ratio (ratio of doses for control and IdUrd pretreated cells at 1% survival) was 1.9 for AB cells and 1.6 for C5 cells. Different symbols for IdUrd curves represent 2 independent experiments.

The present study fails to demonstrate an association of elevated levels of GSH, glutathione transferase, or catalase with radioresistance. It should be noted that the elevated levels of GSH, glutathione transferase, and catalase may contribute to increased resistance to chemotherapy drugs. In particular, modulation of intracellular GSH has been shown to influence chemotherapy cytotoxicity markedly (*14,21,22,29,30*) and to reverse drug resistance in certain multidrug-resistant cells (*14,15*). However, based on the results of the present study and those previously published (*19,31*), GSH depletion would not be expected to greatly influence the response to radiation.

There has been considerable clinical and laboratory interest recently in the rebirth of halogenated pyrimidines as radiosensitizers (*32–34*). Since the pleiotropic drug-resistant C5 cell lines have altered permeability to certain drugs (*4*), experiments were designed to determine if a radiosensitizer such as IdUrd could enter the drug-resistant cells in sufficient concentrations to result in radiosensitization. The C5 cells were clearly made sensitive to radiation by IdUrd incorporation. The extent of radiosensitization was somewhat less in the C5 line than in the parental line. This probably was caused by the longer doubling time of the C5 line, resulting in less replacement by IdUrd for thymidine, compared to the AB line, throughout a 25-hour exposure. The radiosensitization of the drug-resistant cells by IdUrd may offer a viable clinical treatment option for tumors whose proliferation rates and cell-cycle times exceed those of the surrounding normal tissues.

There have been numerous studies directed toward correlations between inherent in vitro radiosensitivity of tumor cell lines and the clinical radiation responsiveness of the associated tumor (*35-41*). In general, few studies correlate clinical radioresistance with the D_0 of the radiation survival curve (*40,41*). However, good correlation between in vitro characteristics such as the ability to repair potentially lethal damage (*39*), large n (*35,38*), high survival values for doses of 2 Gy (*38,40,41*), and the clinical radioresponsiveness of the tumor have been reported. In a recent review of radiation survival curve parameters of 64 in vitro tumor cell lines by Fertil and Malaise (*41*), the surviving fraction at 2 Gy correlated well with clinical responsiveness. It might be instructive to survey the literature to determine what proportion of tumors that are less responsive to radiotherapy were treated with chemotherapy prior to radiation. With respect to radiation treatment of drug-resistant tumors, more data are needed to associate drug resistance with radiation resistance. Clinically, it is not uncommon to see tumors that appear to be resistant to drugs but respond to radiation and vice versa. Whether the response is as "complete" as it might have been without prior treatment with another modality is unclear. In these cases, the selection factors are enormous for physician bias with respect to the size of the tumor, possible alterations of the tumor bed, and performance status.

Presently, there is a tendency for the somatic mutation theory of Goldie and Coldman (*42*) to be embraced as "the" explanation for all drug resistance. While examples of this phenomenon clearly occur, there are other possible explanations for some instances of clinical resistance. These include alterations of tumor bed and vascularity due to prior treatment and changes in the dynamics of tumor growth rate resulting from exposure to agents that do not necessarily destroy a significant or detectable proportion of the cells that comprise tumor mass. The data in the present study suggest that the inherent radiosensitivity of a drug-resistant cell line is not necessarily altered, compared to that of parental lines.

REFERENCES

(*1*) Bech-Hansen NT, Till JE, Ling V: Pleiotropic phenotype of colchicine-resistant CHO cells: Cross resistance and collateral sensitivity. J Cell Physiol 88:23-31, 1976.

(*2*) Ling V: Genetic basis of drug resistance in mammalian cells. *In* Drug and Hormone Resistance in Neoplasia (Bruchovsky N, Goldie JH, eds). Boca Raton, FL: CRC Press, 1982, pp 1-19.

(*3*) Ling V, Kartner N, Sudo T, et al: Multidrug-resistance phenotype in Chinese hamster ovary cells. Cancer Treat Rep 67:869-874, 1983.

(*4*) Ling V, Thompson LH: Reduced permeability in CHO cells as a mechanism of resistance to colchicine. J Cell Physiol 83:103-116, 1974.

(*5*) Kartner N, Riordan JR, Ling V: Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science 221:1285-1288, 1983.

(*6*) Riordan JR, Deuchars K, Kartner N, et al: Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. Nature 316:817-819, 1985.

(*7*) Tietze F: Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Application to mammalian blood and other tissues. Anal Biochem 27:502-522, 1969.

(*8*) Massey V, Williams CH: On the reaction mechanism of yeast glutathione reductase. J Biol Chem 240:4470-4480, 1965.

(*9*) Paglia DE, Valentine WN: Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70:158-169, 1967.

(*10*) Habig WH, Dohat MJ, Jakoby WB: Glutathione *S*-transferases. J Biol Chem 249:7130-7139, 1974.

(*11*) Marklund S, Marklund G: Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469-474, 1974.

(*12*) Bergmeyer H: Zur messung von katalase—aktivitaten. Biochem Z 327:255-258, 1955.

(*13*) Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254, 1976.

(*14*) Suzukake K, Petro BJ, Vistica DT: Reduction in glutathione content of L-PAM resistant L1210 cells confers drug sensitivity. Biochem Pharmacol 31:121-124, 1982.

(*15*) Green JA, Vistica DT, Young RC, et al: Melphalan resistance in human ovarian cancer. Characterization of drug resistant cell lines and potentiation of melphalan cytotoxicity by glutathione depletion. Cancer Res 44:5427-5431, 1984.

(*16*) Louie KG, Behrens BC, Kinsella TJ, et al: Radiation survival parameters of antineoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. Cancer Res 45:2110-2115, 1985.

(*17*) Dethmers JK, Meister A: Glutathione export by human lymph cells: Depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation. Proc Natl Acad Sci USA 78:7492-7496, 1981.

(*18*) Meister A, Anderson ME: Glutathione. Annu Rev Biochem 52:711-760, 1983.

(*19*) Mitchell JB, Russo A, Biaglow JE, et al: Cellular glutathione depletion by diethyl maleate or buthionine sulfoximine: No effect of glutathione depletion on the oxygen enhancement ratio. Radiat Res 96:422-428, 1983.

(*20*) Mitchell JB, Russo A, Kinsella TJ, et al: Elevation in cellular glutathione during thermotolerance induction and thermosensitization by glutathione depletion. Cancer Res 43:987-991, 1983.

(*21*) Russo A, Mitchell JB, McPherson SJ, et al: Alteration of bleomycin cytotoxicity by glutathione depletion or elevation. Int J Radiat Oncol Biol Phys 10:1675-1678, 1984.

(*22*) Russo A, Mitchell JB: Potentiation and protection of doxorubicin cytotoxicity by cellular glutathione modulation. Cancer Treat Rep 69:1293-1296, 1985.

(*23*) Russo A, Mitchell JB: Radiation response of Chinese hamster cells after elevation of intracellular glutathione levels. Int J Radiat Oncol Biol Phys 10:1243-1247, 1984.

(*24*) Russo A, Mitchell JB, DeGraff W, et al: The effects of cellular glutathione elevation on the oxygen enhancement ratio. Radiat Res 103:232-239, 1985.

(*25*) Williamson JM, Boettcher B, Meister A: Intracellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis. Proc Natl Acad Sci USA 79:6246-6249, 1982.

(*26*) Wallner K, Li GC: Adriamycin resistance, heat resistance and radiation response in Chinese hamster fibroblasts. Int J Radiat Oncol Biol Phys 12:829-833, 1986.

(*27*) Terasima R, Tolmach LJ: X-ray sensitivity and DNA synthesis in synchronous populations of HeLa cells. Science 140:490–492, 1963.

(*28*) Biaglow JE, Varnes ME, Tuttle SW, et al: The effect of L-buthionine sulfoximine on the aerobic radiation response of A549 human lung carcinoma cells. Int J Radiat Oncol Biol Phys 12:1139–1142, 1986.

(*29*) Russo A, DeGraff W, Friedman N, et al: Selective modulation of glutathione levels in human normal versus tumor cells and subsequent differential response to chemotherapy drugs. Cancer Res 46:2845–2848, 1986.

(*30*) DeGraff W, Russo A, Mitchell JB: Glutathione depletion greatly reduces neocarzinostatin cytotoxicity in V79 cells. J Biol Chem 260:8312–8315, 1985.

(*31*) Biaglow JE, Morse-Guadio M, Varnes ME, et al: Nonprotein thiols and the radiation response of A549 human lung carcinoma cells. Int J Radiat Biol 44:489–495, 1983.

(*32*) Kinsella TJ, Russo A, Mitchell JB, et al: A phase 1 study of intermittent intravenous bromodeoxyuridine (BUdR) with conventional fractionated irradiation. Int J Radiat Oncol Biol Phys 10:69–76, 1984.

(*33*) Kinsella TJ, Mitchell JB, Russo A, et al: The use of halogenated thymidine analogs as clinical radiosensitizers: Rationale, current status and future prospects: Nonhypoxic cell sensitizers. Int J Radiat Oncol Biol Phys 10:1399–1406, 1984.

(*34*) Mitchell JB, Russo A, Kinsella TJ, et al: The use of non-hypoxic cell sensitizers in radiobiology and radiotherapy. Int J Radiat Oncol Biol Phys 12:1513–1518, 1986.

(*35*) Barranco SC, Romsdahl MM, Humphrey RM: The radiation response of human malignant melanoma cells grown in vitro. Cancer Res 31:830–833, 1971.

(*36*) Weichselbaum RR, Epstein J, Little JB, et al: In vitro cellular radiosensitivity of human malignant tumors. Eur J Cancer 12:47–51, 1976.

(*37*) Weichselbaum RR, Nove J, Little JB: X-ray sensitivity of human tumor cells in vitro. Int J Radiat Oncol Biol Phys 1:621–629, 1981.

(*38*) Carney DN, Mitchell JB, Kinsella TJ: In vitro radiation and chemotherapy sensitivity of established cell lines of human small cell lung cancer and its large cell morphological variants. Cancer Res 43:2806–2811, 1983.

(*39*) Weichselbaum RR: Radioresistant and repair proficient cells may determine radiocurability in human tumors. Int J Radiat Oncol Biol Phys 12:637–639, 1986.

(*40*) Fertil B, Malaise EP: Inherent cellular radiosensitivity as a basic concept for human tumor radiotherapy. Int J Radiat Oncol Biol Phys 1:621–629, 1981.

(*41*) Fertil B, Malaise EP: Intrinsic radiosensitivity of human cell lines is correlated with radioresponsiveness of human tumors: Analysis of 101 published survival curves. Int J Radiat Oncol Biol Phys 11:1699–1707, 1985.

(*42*) Goldie JH, Coldman AJ: A mathematic model for relating the drug sensitivity of tumors to their spontaneous mutation rate. Cancer Treat Rep 63:1727–1733, 1979.

Altered Radiosensitivity of Hematopoietic Stem Cells by Vincristine Pretreatment: Superoxide Dismutase Activity as a Possible Mechanism [1]

Roberta M. Johnke,* Charles J. Kovacs, Dean P. Loven, Reed S. Abernathy, and Jerry L. Hooker [2,3]

ABSTRACT—**The effect of vincristine (VCR) on hematopoietic stem cell and progenitor compartments and its ability to induce transient periods of radioresistance was investigated so that we could ascertain the drug–radiation intertreatment interval affording optimal radioprotection and determine if its ability to induce increased levels of superoxide dismutase (SOD) is a potential mechanism for this radioprotection. Measurement of marrow stem cell and progenitor compartments demonstrated that these subsets displayed differential sensitivity to VCR and that this sensitivity appeared to be proportional to how "primitive" the subset was. Treatment with VCR prior to irradiation was observed to enhance significantly both 8- and 12-day spleen colony-forming unit recovery with maximal radioprotection occurring for a drug-radiation interval of 12–48 hours. Monitoring of copper–zinc SOD levels demonstrated an increase in activity following VCR that was localized in a fraction of the bone marrow enriched for stem cells and progenitors. The temporal pattern of this increase, however, did not correlate with the drug–radiation schedules affording optimal radioprotection, which indicates that other factors appear to be operative in this radioprotection as well.—NCI Monogr 6:193–197, 1988.**

Since the advent of combined modality therapy, significant advancements in the control and cure of neoplasms have been made (*1*). Even more aggressive treatments, however, are needed to achieve tumor cell kill in many cancer patients. Unfortunately, therapeutic protocols must be formulated not only with a consideration of their tumor toxicity but also with an awareness of the tolerance levels of involved normal tissues. Clearly, a major therapeutic advantage could be realized by the development of protocols that result in limited toxicity to normal tissues while still maintaining their antitumor effects. Along these lines, one area of research receiving great attention in recent years concerns radiosensitivity, in which investigators have focused on a pretreatment with various agents, including chemotherapeutics, prior to radiation exposure in an attempt to take advantage of potentially synergistic mechanisms (*2–13*). Unexpectedly, however, this approach has demonstrated that many of these agents, when appropriately administered with radiation, enhance the recovery in normal tissue when compared with treatment with radiation alone. Of equal significance is the failure of these agents, under identical administration schedules, to alter the therapeutic effects on tumor tissue (*14*). This "tissue sparing" effect has been documented for a number of agents, but little is known of the mechanism(s) involved, although several hypotheses have been proposed: 1) recruitment of cells into a relatively radioresistant phase of the cell cycle, 2) increase in the pool of available nucleic acid precursors needed for cellular regeneration, 3) initiation of a feedback loop that induces proliferation in surviving stem cells, and 4) induction of radioprotective and/or repair enzymes (*11–17*).

In the late 1960s, Smith and co-workers (*15,16*) reported that pretreatment with the vinca alkaloids vinblastine and VCR prolonged the survival of mice following radiation by enhancing hematopoietic recovery. Since that time, little emphasis has been focused on the magnitude and mechanism(s) surrounding this potentially important radioprotective effect. Inasmuch as VCR represents a clinically important drug that is frequently administered in combination with radiation in therapeutic regimens, studies were designed to define further the magnitude of the hematopoietic radioprotective potential of VCR and to establish whether the temporal induction of the enzyme SOD following VCR represents a possible mechanism for this radioprotection.

MATERIALS AND METHODS

Experimental animals and treatment.—All investigations were performed on BDF_1 $[(C57BL/6 \times DBA/2)F_1]$ mice. Mice were allowed food and water ad libitum and were maintained on a 12-hour light–dark cycle. Animals were killed by cervical dislocation.

A stathmokinetic agent, VCR is rapidly cleared when injected ip [plasma half-life = 60 min; (*18*)]. For this series of experiments, VCR was dissolved in sterile physiologic

---

ABBREVIATIONS: VCR = vincristine; SOD = superoxide dismutase; CFUs = colony-forming unit(s) in the spleen; CFUs-8d = CFUs assayed at day 8; CFUs-12d = CFUs assayed at day 12; CFUgm = granulocyte/macrophage CFU(s); BFUe = erythroid burst-forming unit(s); CFU-GEMM = granulocyte/erythrocyte/macrophage/megakaryocyte CFU(s); HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H,HBSS = HEPES-buffered Hanks' balanced salt solution with 10% fetal calf serum.

---

[1] Supported by Public Health Service grants CA-42036 and CA-32903 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Division of Radiation Biology and Oncology, The Radiation Oncology Center, East Carolina University School of Medicine, Greenville, NC.

[3] We thank Ms. Anne H. Summerlin, Ms. Jean M. Joiner, Ms. A. Lynn Garrell, and Ms. Deborah L. Downes for assistance and encouragement during this investigation.

* *Reprint requests:* Roberta M. Johnke, Ph.D., Division of Radiation Biology and Oncology, The Radiation Oncology Center, East Carolina University School of Medicine, Greenville, NC 27858-4354.

saline at a concentration of 0.1 mg/ml and injected ip at a dose of 1 mg/kg body weight. This is well below the 50% lethal dose for a 30-day interval for ip injection (4 mg/kg body weight) determined for BDF_1 mice in our laboratory. This dose results in a moderate myelotoxic effect with marrow cellularity decreasing to a nadir of 65%±8% of control at 24 hours post-VCR before recovering to near control levels (89%±8%) by 72 hours.

The physical factors of irradiation were 275 kilovolt peaks, 15 milliamperes, half-value layer=0.55 mm copper plus 0.90 mm aluminum, and a target to source distance of 51 cm. For both the treatment and CFUs assay doses, animals were positioned in acrylic containers without anesthesia and received either 4.5 or 7.5 Gy whole-body exposure, respectively, at a dose rate of 0.6 Gy/min. No animal death was observed at this treatment dose during the time frame of the experiment.

Stem cell and progenitor cell assays.—Marrow cell suspensions were prepared and used in the assay for in vivo CFUs-8d and CFUs-12d and in vitro CFUgm (L-CSA conditioned medium used as the source of colony-stimulating activity) and BFUe (erythropoietin used as the stimulating activity) as previously described (*19*). The CFU-GEMM were assayed by the method of Nakahata and Ogawa (*20*), with erythropoietin and pokeweed mitogen spleen cell conditioned medium used as stimuli.

Fractionation of marrow on Percoll density gradients.—Three dilutions of Percoll (Sigma Chemical Co., St. Louis, MO), i.e., 30%, 55%, and 70% corresponding to densities of 1.055, 1.080, and 1.100 g/cm^3, respectively, were made up in HBSS buffered with 20 m*M* HEPES, containing 10% fetal calf serum and adjusted to a pH of 7.2 (H,HBSS). We made discontinuous gradients by layering 3 ml of 70% Percoll into a conical 15-ml centrifuge tube, overlaying 5 ml of 55% Percoll onto this, and subsequently layering 3 ml of 30% Percoll on top. A 1-ml marrow cell suspension in H,HBSS, from which erythrocytes had been removed by ammonium chloride lysis (*21*), was carefully placed on top of the gradient, and tubes were centrifuged at 600 *g* for 25 minutes. The cells at the 30%/55% interface (low-density fraction) and at the 55%/70% interface (high-density fraction) were collected, resuspended in 10 ml H,HBSS, and centrifuged again for 15 minutes. The pelleted cells were washed once more in H,HBSS and then counted on a hemacytometer. Cells were then aliquoted into tubes at a density of 4–6×10^6 cells, centrifuged, and the pellets frozen (−20° C) until needed for enzymatic assay.

Measurement of copper–zinc superoxide dismutase activity.—Cells from whole bone marrow and the low- and high-density Percoll fractions were resuspended in 1 ml potassium phosphate buffer (50 m*M*, pH 7.8) and sonified with a Tekmar sonic disrupter fitted with a micro tip. Sonification was performed with the machine set on maximal micro tip setting; a 50% pulse time was used for fifteen 1-second pulses with 1-second intervals between the pulses. Samples, kept on ice throughout the sonification, were then brought to 2 ml with additional phosphate buffer, and a 1-ml aliquot was extracted to remove manganese SOD by a chloroform–ethanol extraction method (*22*). Samples were centrifuged at 3,200 *g* and the supernatant stored at −20° C until analysis. The copper–zinc SOD activity was measured by the nitroblue tetrazolium reduction assay (*23*) with some modification (*22,24*). One unit of SOD activity was defined as 50% of maximal inhibition of nitroblue tetrazolium reduction. Results were expressed as units SOD activity per 10^6 cells. Pooled data from different experiments were analyzed according to Dunnett's test for multiple comparisons against one control.

RESULTS

Hematopoietic Response to Vincristine Administration

Hematopoietic subpopulations were assayed following 1 mg VCR/kg for determination of the drug sensitivities of these compartments and their temporal relationships during subsequent recovery (fig. 1). Based on femoral compartment size measured 12 hours after injection, the CFUs-12d was significantly more sensitive to VCR than the other subpopulations measured. Despite its marked sensitivity to VCR at this time, however, initial recovery of the CFUs-12d compartment was rapidly expanding about 10-fold during the subsequent 12-hour interval. A concomitant expansion of CFUs-8d amounted to only a 17% increase, whereas the CFU-GEMM compartment actually decreased. Only slight or insignificant changes were observed in the CFUgm and BFUe compartments at this time. Between 24 and 48 hours, both the CFUgm and BFUe increased to approximately 80% of their predrug control levels; the CFUs-8d and CFUs-12d demonstrated little change. This increase in CFUgm and BFUe at a time when both CFUs populations have essentially leveled off may reflect a movement of these more primitive cells into the more differentiated progenitor cell compartments.

Hematopoietic Response to Vincristine/Radiation

The temporal recovery of the CFUs-8d and CFUs-12d were studied in marrow pretreated at various times with VCR subsequent to receiving 4.5 Gy of x-radiation (−1, −12, −24, −48 hr; fig. 2). The temporal recovery of both CFUs subpopulations was enhanced following combined VCR and radiation with an optimal intertreatment time ranging from 12 to 48 hours. Under these optimal schedules, both the 8d and 12d CFUs had approached control levels by day 21. Following radiation alone, on the other hand, the 8d and 12d CFUs had only repopulated to

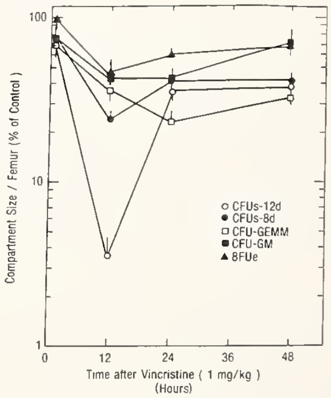


FIGURE 1.—Effect of 1 mg VCR/kg body weight on the hematopoietic stem cell and progenitor cell compartments expressed as a percent of non-drug-treated control values. Each data point represents the mean ± SEM for 3 experiments. Pooled marrow from 4 to 6 mice was used per data point per experiment. ○=CFUs-12d; ●=CFUs-8d; □=CFU-GEMM; ■=CFUgm; ▲=BFUe.

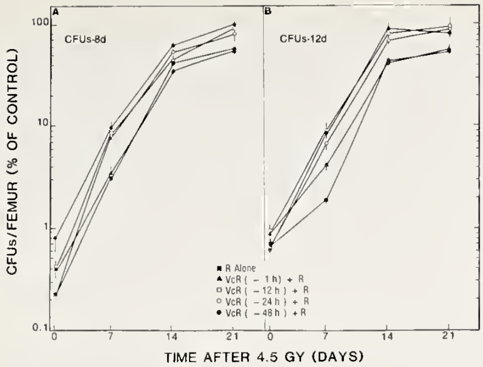


FIGURE 2.—Effect of intertreatment time on the sensitivity of the CFUs-8d (A) and CFUs-12d (B) to combined VCR and radiation expressed as a percent of non-drug-treated control values. Each data point represents the mean ± SEM for 3 experiments. Pooled marrow from a minimum of 5 animals was used per data point per experiment. *Values in parentheses* represent the time before irradiation that VCR was administered. ■ = radiation alone; VCR administered 1 hr (▲), 12 hr (□), 24 hr (○), and 48 hr (●) before irradiation.

approximately 55% of control levels by day 21. The VCR given 1 hour prior to irradiation appeared to have an insignificant radioprotective effect on either CFUs population.

Copper-Zinc Superoxide Dismutase Activity of Fractionated Bone Marrow

In our laboratory, discontinuous Percoll gradients are used routinely for the separation of the bone marrow into two fractions: 1) a low-density fraction containing a 4- to 5-fold enrichment of viable stem and progenitor cells; and 2) a high-density fraction depleted of stem cells and progenitors but enriched in mature marrow elements. Because the stem and progenitor cells are responsible for the recovery of marrow cellularity after radiation insult, and there is sufficient evidence suggesting a possible causal relationship between radiosensitivity and the antioxidant enzymes, SOD activity following VCR was investigated in the Percoll fractions as well as unfractionated bone marrow.

The effect of VCR on copper–zinc SOD activity in bone marrow depleted of mature red blood cells at various times following VCR treatment is summarized in table 1. For the cell populations analyzed, copper–zinc SOD activity was elevated at 1 hour post-VCR and remained elevated through 12 hours in the low-density marrow fraction. However, enzyme activity in both the whole bone marrow and high-density marrow fractions returned to near control levels at this time. By 24 hours after VCR treatment, the enzyme activity in the low-density fraction underwent a second elevation, which was also observed as a more significant peak of activity in the whole bone marrow. During the subsequent 24 hours, the copper–zinc SOD activity in both of these populations returned to near control values. With the exception of the transient 1-hour elevation, the copper–zinc SOD in the high-density fraction remained relatively unchanged throughout the 72-hour interval.

DISCUSSION

Pretreatment with a wide variety of chemotherapeutic agents has been demonstrated to radioprotect normal tissues with the degree of protection observed to be a function of the drug–radiation intertreatment interval (*2–16*). The mechanisms involved in this radioprotective effect are not yet known. However, the time dependency of the phenomenon, its appearance in more than one type of tissue, and the observation that agents displaying a wide range of chemical properties elicit this effect suggest that a common biologic mechanism is involved that is independent of the metabolic pathway of any particular drug. As investigators in this laboratory have reported (*25*) increased radioresistance of intestinal epithelium when VCR was injected 24 hours prior to radiation challenge, the current investigation represents an extension of these studies to the hematopoietic marrow. Collectively, the data reported herein have led to the following conclusions. First, measurement of the marrow stem cell and progenitor compartments following VCR administration (fig. 1) demonstrated that 1) the various subsets of the marrow display differential sensitivities to the drug which appear to be proportional to how "primitive" the subset is, and 2) the recovery of the marrow compartments suggests that a biphasic response to VCR is occurring in the stem cell compartments consisting of an initial phase of marked self-renewal followed by what appears to be a period of reduced self-renewal and increased "feeding" into the more differentiated progenitor cell compartments.

This biphasic recovery of the hematopoietic system following insult has been reported elsewhere (*26,27*) and is consistent with the generation–age hypothesis of tissue renewal (*28*). The pattern of drug-sensitivity, however, is surprising because VCR is considered to be a cycle-specific drug and would be expected to be more toxic to mature populations in which a higher percentage of cells are in cycle. Because uptake of VCR into the cell reportedly involves an active, carrier-mediated process (*29*), it is possible that the differential drug sensitivity pattern observed reflects principally a differential drug uptake potential within the marrow subsets.

TABLE 1.—Effect of VCR on copper–zinc SOD activity in bone marrow[a]

	Time after VCR injection, hr					
Cell population	0	1	12	24	48	72
Whole bone marrow	0.416 ± 0.139 (4)	0.866 ± 0.124 (8)	0.468 ± 0.073 (9)	2.503 ± 0.454 (6)	0.257 ± 0.050 (10)	0.780 ± 0.125 (3)
Low-density fraction	0.535 ± 0.085 (3)	0.921 ± 0.177 (5)	0.966 ± 0.189 (6)	1.777 ± 0.292 (7)	0.449 ± 0.067 (11)	0.237 ± 0.047 (10)
High-density fraction	0.473 ± 0.145 (6)	0.867 ± 0.148 (7)	0.468 ± 0.083 (7)	0.364 ± 0.048 (4)	0.292 ± 0.074 (6)	0.359 ± 0.045 (11)

[a] Values are expressed as units of copper–zinc SOD activity/10^6 cells and are the mean ± SEM for the number of samples *in parentheses*.

Also of considerable interest is the significant expansion of the stem cell compartments between 12 and 48 hours following VCR. If this expansion reflects proliferative activity, one would predict, based on the "law" of Bergonie and Tribondeau (*30*), that these compartments should be more sensitive to radiation at this time, a contradictory observation when one notes that this interval is exactly the time that maximal radioprotection from VCR pretreatment occurs. It seems likely, therefore, that factors other than proliferation are involved. However, exposure to VCR may partially synchronize the stem cell populations such that a large cohort of cells is in a radioresistant phase of the cell cycle at the time points monitored. Furthermore, although the CFUs may indeed be more sensitive to radiation at this time, VCR may precipitate a more rapid recruitment of an even more primitive stem cell compartment that could effectively repopulate the marrow following radiation.

Secondly, whereas our data reaffirm the earlier observations that pretreatment with VCR enhances hematopoietic recovery, the drug–radiation intertreatment interval under which maximal radioprotection occurs appears to be much broader than previously reported. In the earlier report, 48 hours prior to radiation was the optimal time suggested for VCR treatment. However, we find little difference in the radioprotection obtained for VCR given 12, 24, or 48 hours before radiation (radioprotection observed for a 72-hr VCR–radiation interval was significantly less than optimal; data not shown).

Finally, our data indicate that the activity of copper–zinc SOD did increase in the bone marrow (table 1), but the temporal pattern of its increase did not appear to correlate with the kinetics of stem cell radioprotection observed. Little is known concerning the levels of SOD activity in specific populations of the marrow. Recently, 2 studies have suggested that the radiosensitivity of murine bone marrow cells varies inversely with endogenous levels of SOD (*31*) and that the addition of exogenous SOD will radioprotect the CFUgm (*32*). We observed that a transient increase in copper–zinc SOD activity was present at 1 hour post-VCR and that a larger, more pronounced peak occurred at 24 hours afterward that was localized in the low-density (stem cell and progenitor-enriched) fraction. The reasons for the elevated enzyme activity seen at 1 hour postinjection are not clear. However, the rapidity and transient nature of this increase suggest that factors other than enzyme induction are operative including: 1) concentration of a small population with high enzyme content by the selective drug killing of low enzyme-containing cells; or 2) an acute inflammatory reaction from the drug initiating a rapid influx of cells into the marrow that have relatively higher levels of enzyme. For example, the macrophage appears to have much higher SOD activity than does the marrow (10 U/10^6 vs. 0.4 U/10^6 cells). Therefore, a significant influx of macrophages could substantially increase the apparent activity of the marrow. Elevated marrow cellularity was consistently observed at 1 hour postinjection (116% ± 7% of control; $n=5$), which supports the possibility of an influx of cells.

The marked increase in activity at 24 hours more likely reflects enzyme induction. Furthermore, its localization in the fraction enriched for stem and progenitor cells suggests that it could be contributing to the radioprotection of the marrow at this time. Nevertheless, our failure to observe elevated copper–zinc SOD levels at 48 hours after VCR suggests that elevated enzyme levels are an accompanying, rather than a causative, factor in the mechanism responsible for VCR-induced radioprotection. More study will be necessary before the relationship between copper–zinc SOD activity and hematopoietic radioprotection, as well as the radioprotection observed in other tissues, can be fully elucidated.

REFERENCES

(*1*) CARTER SK: Experimental design considerations in clinically combining radiation and drugs. Int J Radiat Oncol Biol Phys 5:1139–1143, 1979.

(*2*) SMITH WW, ALDERMAN IM, GILLESPIE RE: Increased survival in animals treated with bacterial endotoxins. Am J Physiol 191:124–129, 1957.

(*3*) SMITH WW, BRECHER G, FRED S, et al: Effect of endotoxin on the kinetics of hemopoietic colony-forming cells in irradiated mice. Radiat Res 27:710–717, 1966.

(*4*) SMITH WW: Protective effect of a colchicine derivative in mice exposed to x-radiation. Science 127:473, 1958.

(*5*) SMITH WW, ALDERMAN IM, SCHNEIDER CA, et al: Effect of pretreatment with colchicine derivative on hemopoiesis in irradiated mice. Radiat Res 19:621–627, 1963.

(*6*) BRECHER G, SMITH WW, WILSON S, et al: Kinetics of colchicine-induced hemopoietic recovery in irradiated mice. Radiat Res 30:600–610, 1967.

(*7*) ROTHE WE, GRENAN MM: Radioprotection by mitotic inhibitors and mercaptoethylamine. Science 133:888, 1961.

(*8*) COLE LJ, GOSPE SR: Increased radioresistance in mice injected with urethan one day before x-irradiation. Radiat Res 15:684–693, 1961.

(*9*) MILLAR JL, HUDSPETH BN, BLACKETT NM: Reduced lethality in mice receiving a combined dose of cyclophosphamide and busulphan. Br J Cancer 32:193–198, 1975.

(*10*) MILLAR JL, HUDSPETH BN: Sparing effect of cyclophosphamide (NSC-26271) pretreatment on animals lethally treated with γ-irradiation. Cancer Treat Rep 60:409–414, 1976.

(*11*) MILLAR JL, BLACKETT NM, HUDSPETH BN: Enhanced post-irradiation recovery of the haemopoietic system in animals pretreated with a variety of cytotoxic agents. Cell Tissue Kinet 11:543–553, 1978.

(*12*) PHELPS TA, BLACKETT NM: Protection of intestinal damage by pretreatment with cytarabine (cytosine arabinoside). Int J Radiat Oncol Biol Phys 5:1617–1620, 1979.

(*13*) MILLAR JL, STEPHENS TC: An explanation for the ability of cytotoxic drug pretreatment to reduce bone marrow related lethality of total body irradiation (TBI). Int J Radiat Oncol Biol Phys 8:581–583, 1982.

(*14*) MILLAR JL, MCELWAIN TJ: Combinations of cytotoxic agents that have less than expected toxicity on normal tissues in mice. Antibiot Chemother 23:271–282, 1978.

(*15*) SMITH WW, WILSON SM: Effects of vinblastine and vincristine on survival and hemopoiesis in irradiated mice. J Natl Cancer Inst 39:1055–1066, 1967.

(*16*) SMITH WW, WILSON SM, FRED SS: Kinetics of stem cell depletion and proliferation: Effects of vinblastine and vincristine in normal and irradiated mice. J Natl Cancer Inst 40:847–854, 1968.

(*17*) PETKAU A: The role of superoxide dismutase in radiation injury. *In* Superoxide Dismutase (Oberley LW, ed), vol III. Boca Raton, FL: CRC Press, 1985, pp 99–127.

(*18*) EL DAREER SM, WHITE VM, CHEN FP, et al: Distribution and metabolism of vincristine in mice, rats, dogs and monkeys. Cancer Treat Rep 61:1269–1277, 1977.

(*19*) Kovacs CJ, Johnke RM, Emma DA, et al: Residual Adriamycin (AdR)-induced hematopoietic damage: A consideration for subsequent radiotherapy. Int J Radiat Oncol Biol Phys 11:1955–1961, 1985.

(*20*) Nakahata T, Ogawa M: Clonal origin of murine hemopoietic colonies with apparent restriction to granulocyte-macrophage-megakaryocyte (GMM) differentiation. J Cell Physiol 111:239–246, 1982.

(*21*) Mishell BB: Lysis of red blood cells with Tris-buffered ammonium chloride. *In* Selected Methods in Cellular Immunology (Mishell BB, Shiggi SM, eds). San Francisco: Freeman, 1980, p 23.

(*22*) Loven DP, Oberley LW, Rousseau FM, et al: Superoxide dismutase activity in 1,2-dimethylhydrazine-induced rat colon adenocarcinoma. JNCI 65:377–381, 1980.

(*23*) Beauchamp C, Fridovich I: Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287, 1971.

(*24*) Eldred GE, Hoffert JR: A test for endogenous interferences in superoxide dismutase assays. Anal Biochem 110:137–143, 1981.

(*25*) Kovacs CJ: Redefining clinical research. *In* Lung Cancer: Diagnostic Procedures and Therapeutic Management with Special Reference to Radiotherapy (Scarantino CW, ed). New York: Springer-Verlag, 1985, pp 127–167.

(*26*) Chervenick PA, Boggs DR: Patterns of proliferation and differential of hematopoietic stem cell after compartment depletions. Blood 37:568–580, 1971.

(*27*) Boggs SS, Chervenick PA, Boggs DR: The effect of post-irradiation bleeding or endotoxin on proliferation and differentiation of hematopoietic stem cells. Blood 40: 375–389, 1972.

(*28*) Rosendaal M, Hodgson GS, Bradley TR: Organization of the haematopoietic stem cells: The generation-age hypothesis. Cell Tissue Kinet 12:17–29, 1979.

(*29*) Goldenberg GJ, Begleiter A: Alterations of drug transport. *In* Antitumor Drug Resistance (Fox BW, Fox M, eds). New York: Springer-Verlag, 1984, pp 241–298.

(*30*) Bergonie J, Tribondeau L: Interpretation de quelques resultats de la radiotherapie et essai de fixation d'une technique rationnelle. C R Acad Sci 143:983–985, 1906.

(*31*) Petkau A, Chelak WS, Kelly K, et al: Superoxide dismutase and radiosensitivity: Implications for human mammary carcinomas. *In* Pathology of Oxygen (Autor AP, ed). New York: Academic Press, 1982, pp 223–243.

(*32*) Chelak WS, Petkau A: Identifying superoxide-sensitive progenitor cells in mouse bone marrow. Int J Radiat Biol 44:523–529, 1983.

Inhibition of Pentose Cycle of A549 Cells by 6-Aminonicotinamide: Consequences for Aerobic and Hypoxic Radiation Response and for Radiosensitizer Action [1]

Marie E. Varnes [2,3,*]

ABSTRACT—**Metabolism of glucose via the pentose cycle is a principal source of NADPH, an important cellular reducing species. Both aerobic and hypoxic irradiation stimulate the pentose cycle activity of A549 human lung carcinoma cells, which indicates that NADPH is utilized during irradiation, either as a direct hydrogen donor or as a cofactor for enzymatic repair of radiation damage. To evaluate the role of the pentose cycle in radiation response, we treated A549 cells with 6-aminonicotinamide (6-AN), which blocks the oxidative limb of this pathway in some cell lines. We found 6-AN to be a very effective inhibitor of pentose cycle activity, as indicated both by accumulation of 6-phosphogluconate in A549 cells and by the inability of nitrofurazone or peroxide to stimulate release of $^{14}CO_2$ from ^{14}C-1-labeled glucose after 6-AN treatment. Effects of 6-AN were time and concentration dependent; it caused partial inhibition of glycolysis but had no effect on respiratory rate or on intracellular glutathione levels. Effects of 6-AN on radiation response were examined under two conditions: 1) after treatment with 0.3 m*M* drug for 5 hours, which inhibited pentose cycle activity by 50%, and 2) after treatment for 15 hours, which completely inhibited pentose cycle activity. Neither treatment affected aerobic radiation response, but both increased hypoxic sensitivity to a similar extent, with the oxygen enhancement ratio reduced from 3.0 to 2.0 at a 0.05 surviving fraction. Treatment of A549 cells with 6-AN caused an increase in hypoxic cell radiosensitization by misonidazole, but effects of the combined agents were not more than additive. These studies indicate that NADPH turnover, via the pentose cycle, may be more important for hypoxic than for aerobic radioresponse. However, inasmuch as there was no correlation between the extent of pentose cycle inhibition and reduction of the oxygen enhancement ratio, the results also indicate that A549 cells may utilize other reducing species or other pathways to generate NADPH when the pentose cycle is inhibited.—NCI Monogr 6:199–203, 1988.**

Metabolism of glucose via the PC (fig. 1) supplies the cell with the reducing species, NADPH, as well as with ribose-5-phosphate for nucleic acid and NAD^+ synthesis. Biaglow et al. (*1*) have proposed that PC activity could be important for radiation response because NADPH is a good hydrogen donor and because it is a cofactor for both glutathione reductase and ribonucleotide reductase. Support for this hypothesis comes from studies of Westerman and coworkers (*2*) showing that red cells deficient in glucose-6-phosphate dehydrogenase, a PC enzyme, are much more sensitive to irradiation than are normal cells, and from studies of Roberts et al. (*3*) showing that the PC activity of human lymphocytes is stimulated by aerobic and hypoxic irradiation. It may or may not be circumstantial that several hypoxic cell radiosensitizers, including the nitro compounds and diamide, have been shown to oxidize intracellular NADPH (*4–6*).

To evaluate the role of the PC in radiation response of human lung tumor cells, we used the metabolic analog 6-AN, which blocks PC activity in vitro and in vivo by inhibiting 6-phosphogluconate dehydrogenase [(*7–9*), fig. 1]. This report deals with the effects of 6-AN treatment of A549 cells on both aerobic and hypoxic radiation response, on radiosensitization by misonidazole, and on several aspects of cellular metabolism.

MATERIALS AND METHODS

Cell culture.—Obtained from the American Type Culture Collection (Rockville, MD), A549 human lung carcinoma cells were grown as monolayers in McCoy's 5A medium, supplemented with 10% calf serum and 5% fetal calf serum, and buffered with 2.0 g bicarbonate/liter and 0.02 *M* HEPES, pH 7.2. Cells had a doubling time of 20 ± 2 hours and were used in mid-log phase of growth.

Irradiation protocol.—Cells were harvested by trypsinization and resuspended in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, pH 7.2, at a density of approximately 2×10^5 cells/ml. For aerobic irradiations, 3 ml of a cell suspension were pipetted into 25-cm^2 plastic culture flasks and irradiated at 180 rad/minute with a 250-kilovolt peak Picker-Vanguard x-ray unit. Flasks were placed on a thermostated 37° C rotary shake table and rotated at a moderate speed so that adequate oxygenation was assured. For hypoxic irradiations, 3 ml of a cell suspension were pipetted into glass 25-cm^2 culture flasks. The flasks were sealed with rubber stoppers, inlet and outlet needles were inserted, and flasks were gassed for 15 minutes with 100% N_2 at a flow rate of 1 liter/minute. After gassing, needles were withdrawn, and the flasks were irradiated as described above. Misonidazole, when used, was added to cell suspensions

---

ABBREVIATIONS: PC = pentose cycle; 6-AN = 6-aminonicotinamide; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

---

[1] Supported by Public Health Service grants CA-40516 and CA-13747 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Division of Biochemical Oncology, Department of Radiology, Case Western Reserve University, Cleveland, OH.

[3] I thank Mr. Keith Freeman and Ms. Caroline Gray for technical assistance, Dr. John E. Biaglow for his many helpful discussions of this research, and the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, for the misonidazole.

* *Reprint requests:* Marie E. Varnes, Ph.D., Division of Biochemical Oncology, Department of Radiology, Case Western Reserve University, Wearn Bldg., B-21, Cleveland, OH 44106.

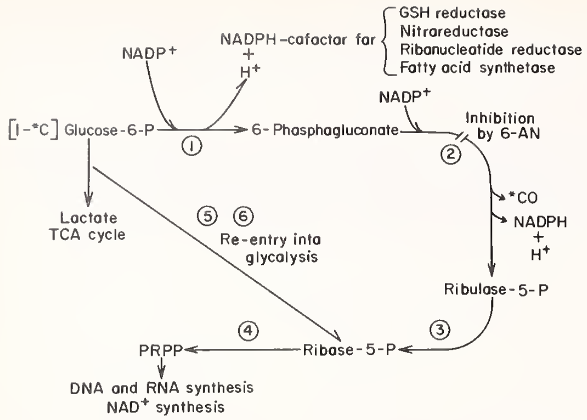


FIGURE 1.—Schematic diagram of the PC. Enzymes are 1) glucose-6-phosphate (glucose-6-P) dehydrogenase; 2) 6-phosphogluconate dehydrogenase; 3) phosphopentose isomerase; 4) ribose phosphate pyrophosphokinase; 5) transaldolase; and 6) transketolase. After being converted to 6-ANADP$^+$, 6-AN inhibits 6-phosphogluconate dehydrogenase (*7*). Formed via the PC as well as other pathways, NADPH is a cofactor for many reducing enzymes, some of which are shown. GSH = glutathione; TCA = trichloroacetic acid; ribose-5-P = ribose-5-phosphate.

just prior to gassing. Clonogenic survival assays were performed as described (*10*).

For the data illustrated in figure 2, cells were trypsinized, resuspended in buffered saline (130 m*M* NaCl, 12.8 m*M* KCl, 0.65 m*M* $MgSO_4$, 0.02 *M* phosphate, and 0.02 *M* HEPES, pH 7.3) containing 2 m*M* labeled glucose, and irradiated with a cobalt-60 gamma source at 1,200 rad/minute.

Biochemical assays.—As we described (*6*), PC activity in intact cells was measured as release of $^{14}CO_2$ from ^{14}C-1 glucose. Cellular levels of 6-phosphogluconate were measured on perchloric acid extracts according to the enzymatic procedure of Haid (*11*), and glycolytic activity of control and 6-AN-treated cells was determined by the monitoring of changes in glucose concentrations in the buffer overlying cells, as described (*6,12*). Intracellular glutathione levels were determined with the Tietze assay (*10*), and respiratory rates, as well as rates of potassium cyanide-insensitive, nitrofurazone-induced oxygen consumption, were measured with the Clark oxygen electrode (*4*).

The 6-AN was used without further purification.

RESULTS

We (*6*) have demonstrated that the hypoxic cell sensitizers misonidazole, SR-2508, and nitrofurazone stimulate the PC activity of A549 cells, as measured by an increased rate of release of $^{14}CO_2$ from ^{14}C-1 glucose. Figure 2 shows that PC activity of these cells can also be stimulated by aerobic and hypoxic irradiation. For this experiment, the $^{14}CO_2$ accumulated during and in the 30 minutes after irradiation was monitored. Results represent a typical experiment; variation in the amount of $^{14}CO_2$ released per gray was about 30% among several experiments. It is notable that the effects of irradiation on PC activity are small relative to the effects of nitrofurazone (table 1). In addition, 5 m*M* misonidazole produced about 18 nmol $^{14}CO_2 \cdot 10^6$ cells·30 minutes (data not shown).

Figure 3 shows the effects of incubation of A549 cells with 0.3 m*M* 6-AN in culture medium on PC activity and on overall glucose consumption. Because 6-AN is a reversible inhibitor of 6-phosphogluconate dehydrogenase (*7*), its inhibitory effect cannot be measured with the use of cell extracts in the presence of added NADPH. Thus we monitored effects of the drug on nitrofurazone-stimulated PC activity, using intact cells. It had been reported that in some cell types treatment with 6-AN results in intracellular accumulation of 6-phosphogluconate (*7,8*); this is true for A549 cells (table 1). Increased levels of 6-phosphogluconate could inhibit glycolysis both by product inhibition (*7*) and possibly by acidification of intracellular pH (*13*). Inhibition of glucose consumption parallels inhibition of the PC (fig. 3). Because less than 4% of glucose consumed by log-phase A549 cells is metabolized via the PC (*6*), the observed effect on overall glucose consumption is not merely a reflection of inhibition of the PC.

The data given in figure 3 indicate that blocking of PC activity of A549 cells completely and specifically with 6-AN would not be possible. Therefore, we used two conditions for radiation studies: 5-hour treatment with 0.3 m*M* 6-AN, which resulted in approximately 50% inhibition of the PC and only 30% inhibition of glucose consumption, and 15-hour treatment with 6-AN, which resulted in nearly complete inhibition of the PC and about 70% inhibition of glucose consumption. Other metabolic effects of 6-AN treatment are shown in table 1. It is interesting that, though 6-AN treatment reduced plating efficiency, it did not alter intracellular glutathione levels nor inhibit respiration. In addition to the metabolic effects shown, a growth delay was

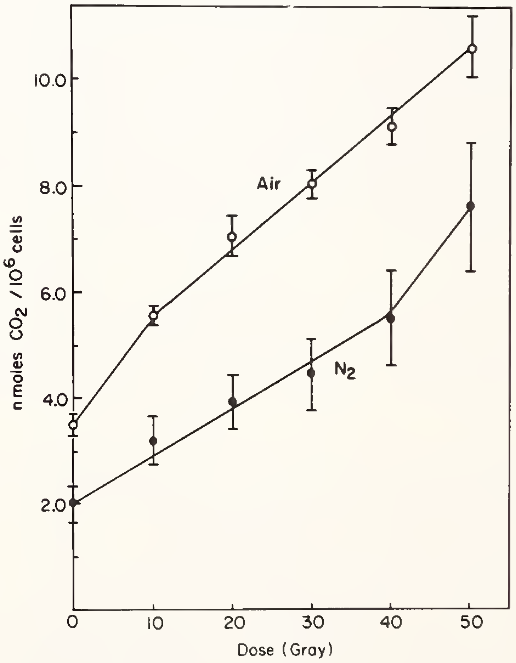


FIGURE 2.—Stimulation of PC activity of A549 cells by aerobic and hypoxic irradiation. Results are averages of duplicate measurements from a typical experiment ± SEM.

TABLE 1.—Metabolic effects of treatment of A549 cells with 6-AN[a]

Assay	Variable measured[b]	Control	6-AN 5-hr	6-AN 15-hr
PC activity	Amount of $^{14}CO_2$ released upon addition of 0.5 mM nitrofurazone to cells (aerobic), nmol/30 min	71 ±5	53 ± 4	2.8±0.3
Glucose utilization	Amount used, nmol/30 min	148 ±6	107 ±11	49 ±4
6-Phosphogluconate in cells	Amount in nmol	1.3±0.5	5.1± 0.4	19.2±1.8
Cellular reducing capacity	Amount of oxygen consumed, nmol/min, upon addition of 0.5 mM nitrofurazone to potassium cyanide-inhibited cells	3.8±0.1	1.9± 0.3	2.2±0.2
Intracellular glutathione	Amount in nmol	41 ±1.0	ND[c]	40 ±1.0
Respiratory rate	Amount of oxygen consumed, nmol/min	4.0±0.3	ND	3.9±0.2
Plating efficiency, %	Colonies/No. of cells plated	59 ±4	42 ± 3	26 ±2

[a] Values represent averages of 2 or more experiments ± SE.

[b] Cells numbered 10^6, except for plating efficiency.

[c] ND = not determined.

observed when 6-AN-treated cells were plated for clonogenic survival.

The radiation response of A549 cells treated for 5 or 15 hours with 0.3 mM 6-AN is shown in figure 4. For these experiments, 6-AN was removed just prior to irradiation, as cells were trypsinized and irradiated as single cell suspensions. Treatment of cells with 6-AN had no effect on aerobic radiation response but did increase hypoxic sensitivity, with reduction of the oxygen enhancement ratio from 3.0 to 2.0 (0.05 surviving fraction). Five- and 15-hour treatments with 6-AN gave similar effects, reducing the shoulder and slightly increasing the slope of the dose–response curve. When 1 mM misonidazole was added to cell suspensions 15 minutes before irradiation, it reduced the oxygen enhancement ratio of untreated cells to 2.0. Treatment of cells with 6-AN for 5 and 15 hours increased the effect of misonidazole in a dose-dependent manner, with nearly additive effects of the two agents at 20 Gy. The enhancement ratios in the presence of misonidazole were 1.9 and 1.8 for the 5- and 15-hour 6-AN treatments, respectively. In the latter case, the oxygen enhancement ratio reflects an increase in aerobic sensitization in the presence of misonidazole. Misonidazole was not toxic to untreated or to 6-AN-treated cells for the short times it was used.

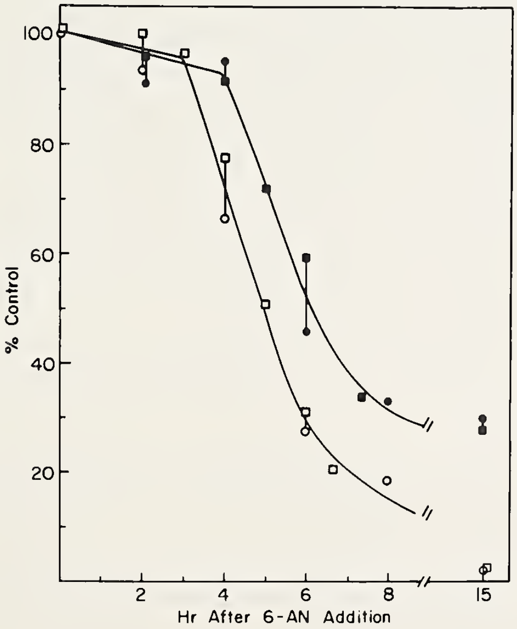


FIGURE 3.—Metabolic effects of incubation of A549 cells with 0.3 mM 6-AN for various times. Nitrofurazone-stimulated PC activity (*open symbols*) and rates of overall glucose consumption (*closed symbols*) were determined as described in Materials and Methods. Results are averages from 2 experiments ± SEM. Control values are presented in table 1.

DISCUSSION

Stimulation of release of $^{14}CO_2$ from ^{14}C-1 glucose by aerobic and hypoxic irradiation (fig. 2) indicates that NADPH is utilized by A549 cells in response to radiation damage, either as a direct hydrogen donor or as a cofactor for enzymes such as glutathione reductase and ribonucleotide reductase (fig. 1). The small effect of irradiation, relative to effects of nitro compounds, would be expected on the basis of G[4] values for oxidizing radicals and peroxide (*14*). Inasmuch as the PC is the chief source of NADPH in many cell lines and the arguments outlined above suggest a role for the PC in radiation response, the data of figure 2 led us to an effort to use metabolic inhibitors of PC activity to elucidate this role further.

Two compounds reported to inhibit the oxidative (NADPH-producing) limb of the PC are the antimetabolite 6-AN (*7,8*) and the steroid hormone dehydroepiandrosterone (*15*). Metabolized to 6-ANADP$^+$ in cells, 6-AN has been shown to inhibit preferentially 6-phosphogluconate dehydrogenase (*7*), whereas the steroid hormone inhibits glucose-6-phosphate dehydrogenase activity in many cell lines (*15*). With A549 cells, dehydroepiandrosterone produced a maximal 30% inhibition of glucose-6-phosphate dehydrogenase as measured on cell extracts, but it failed to

---

[4] The G value is the number of molecules produced per 100 electron volts of energy absorbed by the solution. It has been determined that 1 G = 1.038 μM radiolysis product per 1,000 rad.

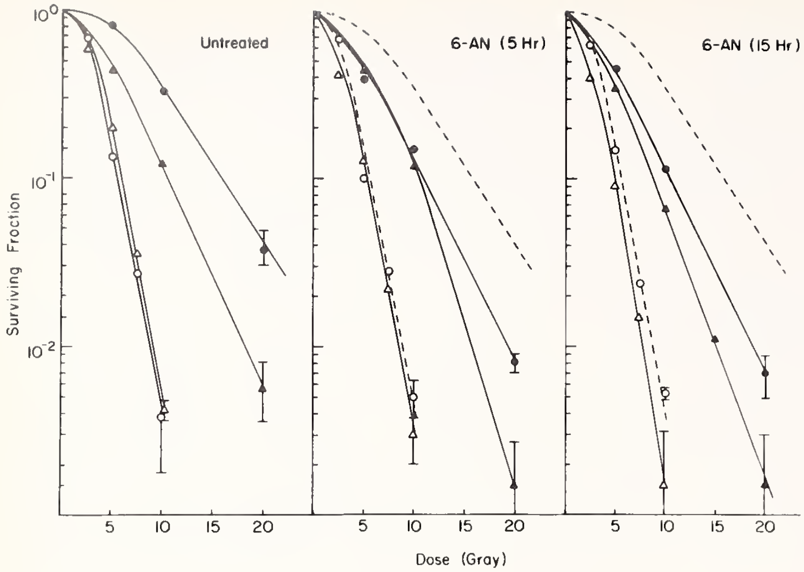


FIGURE 4.—Effect of treatment of A549 cells with 0.3 m*M* 6-AN for 5 or 15 hr on response to ionizing radiation and to the hypoxic cell sensitizer misonidazole. Cells were irradiated as described in Materials and Methods under either aerobic (*open symbols*) or hypoxic (*closed symbols*) conditions. (○, ●) no misonidazole; (△, ▲), 1 m*M* misonidazole added 15 min prior to and present during irradiation. Results are averages of two or more survival curves for each condition ± SEM.

inhibit drug-induced stimulation of the PC in intact cells (data not reported). Therefore, we selected 6-AN as the agent of choice to examine the role of the PC in radiation response of A549 cells.

Figure 3 shows that partial inhibition of glycolysis follows inhibition of the PC by 6-AN. This is most likely a consequence of the buildup of 6-phosphogluconate (table 1), which is a competitive inhibitor of glucose phosphate isomerase (*8*). Others (*8,9*) have observed additional effects of 6-AN treatment. However, it is significant that in A549 cells even complete inhibition of stimulatable PC activity did not affect energy metabolism via respiration nor cause a reduction of endogenous glutathione levels (table 1).

Inhibition of the PC by 6-AN affects hypoxic but not aerobic radiation response, even though PC activity is stimulated by both aerobic and hypoxic irradiation (fig. 4). This suggests that NADPH plays a different role under the two conditions. Currently, we are investigating whether stimulation of the PC by aerobic irradiation is due primarily to the use of NADPH as a cofactor for glutathione reductase (*16*). Figure 4 also shows that radiation response and effect on oxygen enhancement ratio are the same regardless of whether 6-AN causes partial or complete inhibition of the PC. In addition, ability of A549 cells to reduce nitrofurazone is inhibited by about 50% regardless of the extent of PC inhibition (table 1). These results indicate that A549 cells utilize other reducing species or other pathways to generate NADPH when the PC is inhibited. Sufficient NADPH or NADH may be supplied via the malic enzyme or citrate dehydrogenase, at least under aerobic conditions. It is notable that, in contrast to these results, Berger et al. (*9*) observed aerobic radiosensitization of L1210 cells as a result of 6-AN treatment.

Misonidazole is a radiosensitizer that uses NADPH in the course of its metabolic reduction and thus stimulates PC activity of cells under both aerobic and hypoxic conditions (*6*). If, as suggested by Greenstock (*17*), damage to DNA is affected by cell redox status, then inhibition of regeneration of oxidized NADPH might be expected to increase the radiosensitizing effects of misonidazole. Misonidazole and 6-AN produce additive effects on hypoxic radiation response at 20 Gy (fig. 4), even though the biochemical data presented in table 1 indicate that the reducing capacity of A549 cells has not been fully stressed by even 15 hours of 6-AN treatment. We believe it would be worthwhile to test the effects of stressing this capacity further, e.g., by depleting intracellular glutathione or using reducing agents more potent than misonidazole.

REFERENCES

(*1*) Biaglow JE, Varnes ME, Clark EP, et al: The role of thiols in cellular response to radiation and drugs. Radiat Res 95:437–455, 1983.

(*2*) Westerman MP, Wald N, Diloy-Puray M: Irradiation shortens the survival time of red cells deficient in glucose-6-phosphate dehydrogenase. Radiat Res 81:473–477, 1980.

(*3*) Roberts W, Kartha M, Sagone A: Effect of irradiation on the hexose monophosphate shunt pathway of human lymphocytes. Radiat Res 79:601–610, 1979.

(*4*) Biaglow JE: Cellular electron transfer and radical mechanisms for drug metabolism. Radiat Res 86:212–242, 1981.

(*5*) Harris JW: Cellular thiols in radiation and drug response: Use of specific reagents. *In* Radioprotectors and Anticarcinogens (Nygaard OF, Simic MG, eds). New York: Academic Press, 1983, pp 255–274.

(*6*) Varnes ME, Tuttle SW, Biaglow JE: Nitroheterocycle metabolism in mammalian cells: Stimulation of the hexose monophosphate shunt. Biochem Pharmacol 33:1671–1677, 1984.

(*7*) Herken H: Antimetabolic action of 6-aminonicotinamide on the pentose phosphate pathway in the brain. *In* Mechanisms of Toxicity (Alridge WN, ed). London: Macmillan, 1971, pp 189–203.

(*8*) Hunting D, Gowans B, Henderson JF: Effects of 6-aminonicotinamide on cell growth. Poly (ADP-ribose) synthesis and nucleotide metabolism. Biochem Pharmacol 34:3999–4003, 1985.

(*9*) Berger NA, Catino DM, Vietti TJ: Synergistic antileukemic effect of 6-aminonicotinamide and 1,3-bis(2-chloroethyl)-1-nitrosourea on L1210 cells in vitro and in vivo. Cancer Res 42:4382–4386, 1982.

(*10*) Biaglow JE, Clark E, Epp E, et al: Nonprotein thiols and the radiation response of A549 human lung carcinoma cells. Int J Radiat Biol 44:489–495, 1983.

(*11*) HAID E: D-Gluconate-6-phosphate. *In* Methods of Enzymatic Analysis (Bergmeyer HU, ed). New York: Academic Press, 1974, pp 1248–1250.

(*12*) VARNES ME, BIAGLOW JE: Inhibition of glycolysis of mammalian cells by misonidazole and other radiosensitizing drugs: Prevention by thiols. Biochem Pharmacol 31: 2345–2351, 1982.

(*13*) CECCARINI C, EAGLE H: pH as a determinant of cellular growth and contact inhibition. Proc Natl Acad Sci USA 68:229–233, 1971.

(*14*) SPINKS JWT, WOODS RJ: An Introduction to Radiation Chemistry. New York: Wiley, 1963, p 258.

(*15*) OERTEL GW, REBELEIN I: Effect of dehydroepiandrosterone and its conjugates upon the activity of glucose-6-phosphate dehydrogenase in human erythrocytes. Biochim Biophys Acta 184:459–460, 1969.

(*16*) VARNES ME, BIAGLOW JE: Role of peroxidase in stimulation of the pentose cycle of A549 cells by aerobic irradiation. *In* Anticarcinogenesis and Radiation Protection (Simic MG, Nygard OF, eds). New York: Plenum Press, 1987. In press.

(*17*) GREENSTOCK CL: Redox processes in radiation biology and cancer. Radiat Res 86:196–211, 1981.

Radioresistance of Human Tumor Xenografts: Possible Mechanisms [1]

Shirley Lehnert[2,*] **and Marcelle Guichard**[3,4]

ABSTRACT—**Cells of three human tumors irradiated in situ in athymic nude mice are more radioresistant at all doses than are corresponding cells irradiated in vitro. The tumors investigated were Na11 melanoma and two colorectal adenocarcinomas, HRT18 and HT29. While the Na11 tumor contains an exceptionally large hypoxic fraction, this is not true for the other two tumors, and other mechanisms have been proposed to explain these findings. Results of experiments described here suggest that the effect is not dependent on intercellular contact or on the age distribution of the cells in vivo. Tumor cells irradiated in situ were sensitized by both high-pressure oxygen and misonidazole, and the effect of the two agents together was greater than that of either used alone. It is concluded from the shape of the survival curve and from the response to high-pressure oxygen and/or misonidazole that the tumor cell population contains cells ranging from acutely hypoxic to fully oxygenated and includes a subpopulation of partially hypoxic cells of intermediate radiosensitivity.—NCI Monogr 6:205–209, 1988.**

Studies of the radiation response of human tumor cell lines transplanted into athymic nude mice are described in this report. The three tumors that have been investigated are Na11 melanoma and two colorectal carcinomas, HRT18 and HT29; they have been found to be more radioresistant when irradiated in situ, even at low doses, than are the corresponding cells irradiated in vitro (*1,2*). For HRT18 and HT29, this response cannot be explained on the basis of a large hypoxic fraction, so other mechanisms that might explain the discrepancy between the radiation response of cells irradiated in vitro and in vivo have been investigated.

MATERIALS AND METHODS

Tumor cell lines.—The tumors studied were Na11 melanoma, HRT18 rectoadenocarcinoma, and HT29 colonic adenocarcinoma. These tumor cell lines were established from biopsy material, and their characteristics have been described elsewhere (*2,3*). Tumor cells were maintained in vitro in monolayer culture by serial subculture in minimum essential medium containing Earle's salts with 20% fetal calf serum and antibiotics. Female, congenitally athymic nude mice arising from the cross of heterozygous nu/+ females and homozygous nu/nu males were used for implantation of tumors. Mice aged 2–4 months were exposed to 5-Gy total body irradiation 48 hours before sc injection of 3×10^6 tumor cells into the flank.

Cell-survival assay.—Tumors were used when they reached a mean diameter of 8–10 mm, which was 3–4 weeks after implantation of Na11 and HT29 and 8 weeks after implantation of HRT18. The tumors were irradiated in situ and excised approximately 10 minutes later. The preparation of the tumor cell suspension has been described elsewhere (*2*). The excised tumor was cut into small fragments, which were dissociated in 0.1% trypsin for 20 minutes at 37° C with stirring. Cell clumps were removed by filtration; trypsinization was terminated by the addition of serum; and cells collected by centrifugation were resuspended in complete culture medium. An appropriate number of cells were plated in the culture medium for colony-forming assay. Flasks were fixed and stained after 14 days' growth at 37° C, and colonies of ≥ 50 cells were scored as survivors. Colony counts from two to three flasks were averaged for each data point. The control plating efficiencies for cells derived from tumors were $25.2\% \pm 3.5\%$ for HRT18, $10.1\% \pm 3.1\%$ for Na11, and $20\% \pm 4.5\%$ for HT29 (mean ± SD).

Survival curves were drawn by performing a least-squares fit of data in the high-dose exponential region of the curve (surviving fraction <0.1). The low-dose portion of the curve was fitted by eye.

Misonidazole.—Misonidazole was obtained from Roche Products, Ltd. (Welwyn Garden City, England). It was dissolved in saline, and 1 mg/g was injected ip in a volume of 0.5 ml. The timing of drug injection was such that the end of the irradiation time coincided with 60 minutes after drug injection. Control mice were injected with saline, using a similar schedule.

High-pressure oxygen.—Unanesthetized, restrained mice were exposed to HPO at a final pressure of 3.5 atmospheres in a hyperbaric chamber. For irradiation, the chamber was positioned inside a cesium irradiator. Including irradiation time, mice were exposed to 3.5 atmospheres of O_2 for 30 minutes.

Irradiation.—Air-breathing, unanesthetized, tumor-bearing mice were irradiated with ^{137}Cs rays at a dose rate of

---

ABBREVIATION: HPO = high-pressure oxygen.

[1] Supported in part by Ligue Nationale Contre le Cancer (Departement des Hauts-de Seine).

[2] Department of Radiation Oncology, McGill University, Montreal, Quebec, Canada.

[3] Unite INSERM 247, Institut Gustave Roussy, Villejuif, France.

[4] S. Lehnert is grateful for the hospitality and support afforded during a 3-month stay at the Laboratoire de Radiobiologie Cellulaire (Unite INSERM 247) at the Institut Gustave Roussy. We also thank Patrick Ardoine and Valerie Frascogna for acquisition and care of the animals; Françoise Lespinasse for technical assistance; and Joelle Encinas for secretarial help.

* *Reprint requests:* Shirley Lehnert, Ph.D., Department of Radiation Oncology, Montreal General Hospital, 1650 Cedar Ave., Montreal, Quebec H3G 1A4, Canada.

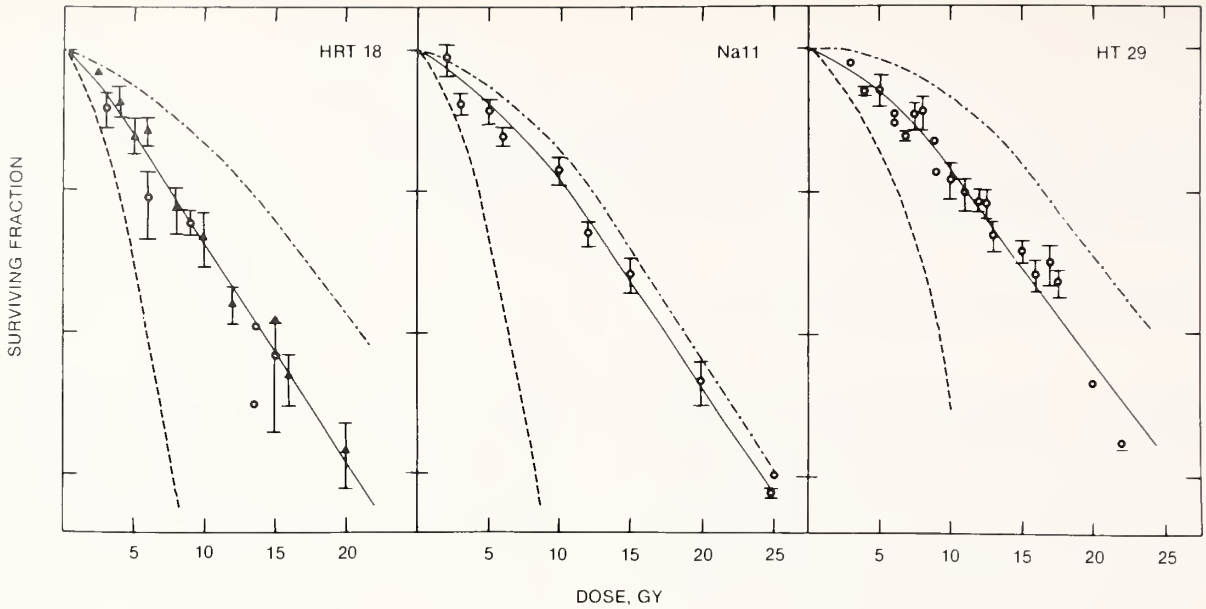


FIGURE 1.—Response of cells from tumors irradiated in situ. – – – =fully oxygenated cells irradiated in vitro. —— =cells irradiated in air-breathing mice. For HRT18, ○ and ▲ represent results of 2 separate sets of experiments. —·— =cells irradiated in mice killed by nitrogen asphyxiation.

1.96 Gy/minute. In some experiments, tumor-bearing mice were killed by nitrogen asphyxiation 15 minutes prior to irradiation.

RESULTS

Survival curves obtained for tumor cells irradiated in situ are shown in figure 1. For all three tumor cell lines, cells irradiated in situ in air-breathing animals were more radioresistant at all dose levels than fully oxygenated cells irradiated in vitro. Survival curves for tumor cells irradiated in air-breathing mice were also compared with survival curves for cells irradiated in nitrogen-asphyxiated mice. In all cases, the slope of the terminal portions of the curve for air-breathing mice was similar to that for cells irradiated under hypoxic conditions. The D_0 values calculated from these curves for tumor cells for air-breathing and nitrogen-asphyxiated mice, respectively, were 2.8 and 3.0 Gy for HRT18, 2.9 and 2.8 Gy for Na11, and 3.2 and 3.3 Gy for HT29.

Figure 2 shows results of experiments in which tumor

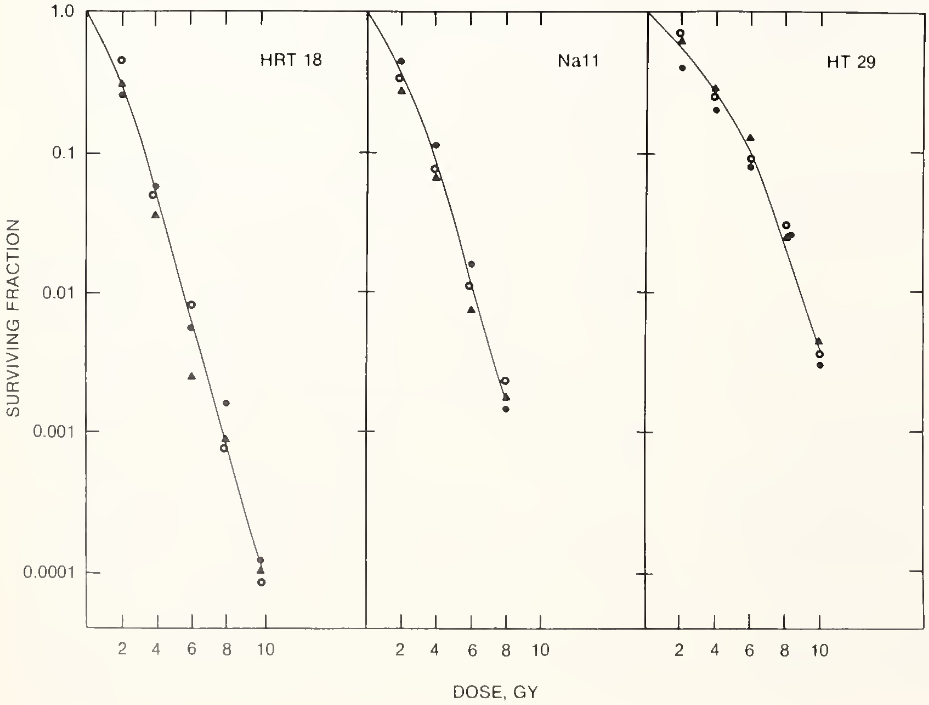


FIGURE 2.—Response of tumor cells irradiated in vitro immediately after plating (○); 5 hr after plating (●); or 24 hr after plating (▲).

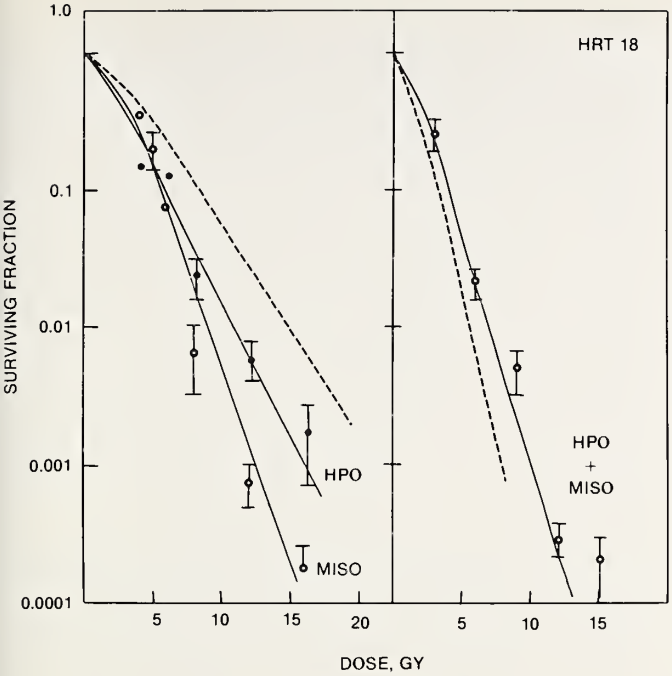


FIGURE 3.—Response of HRT18 tumor cells irradiated in situ following injection of 1 mg of misonidazole/g and/or under HPO. *Left panel:* ——=misonidazole (MISO) or HPO; — — —=control (saline injected). *Right panel:* ——=misonidazole+HPO; — — —=fully oxygenated cells in vitro.

cells were irradiated in vitro. Tumors were excised, a single-cell suspension was prepared by the usual procedure, and cells were irradiated at 0, 5, and 24 hours after plating. There was no detectable difference in radiation sensitivity for cells irradiated at these different times after isolation from the tumor. For all three tumors, survival curves were similar to those obtained when cells derived from tissue culture are irradiated.

In subsequent experiments, tumors were irradiated in situ following injection of misonidazole and/or during exposure to HPO. For the HRT18 tumor (fig. 3), both agents produced a dose-modifying increase in radiosensitivity, which was manifested as a change in slope in the terminal portion of the survival curve. Misonidazole sensitized cells to a greater extent than did HPO. The D_0 values for untreated, HPO-treated, and misonidazole-treated tumors were 2.83, 2.39, and 1.91 Gy, respectively, while the extrapolation number remained the same. Exposure to both HPO and misonidazole at the time of irradiation produced further radiosensitization. Again, the effect was dose modifying, with reduction of the D_0 to 1.39 Gy. This survival curve is similar to, but does not exactly reproduce, the curve obtained when cells are irradiated under aerated conditions in vitro.

For the Na11 tumor (fig. 4), misonidazole alone and misonidazole combined with HPO had a dose-modifying effect, increasing the slope of the survival curve, with no change in the extrapolation number. Combined treatment was more effective than misonidazole alone. However, the combined treatment did not sensitize the cells to the level observed for fully oxygenated cells irradiated in vitro.

For the HT29 tumor (fig. 5), both HPO and misonidazole were again dose modifying. The D_0 values for untreated, HPO-treated, and misonidazole-treated HT29 cells irradiated in situ were 3.2, 2.9, and 2.4 Gy, respectively. Simultaneous exposure to HPO and misonidazole at the time of irradiation produced further sensitization; the D_0 was reduced to 1.9.

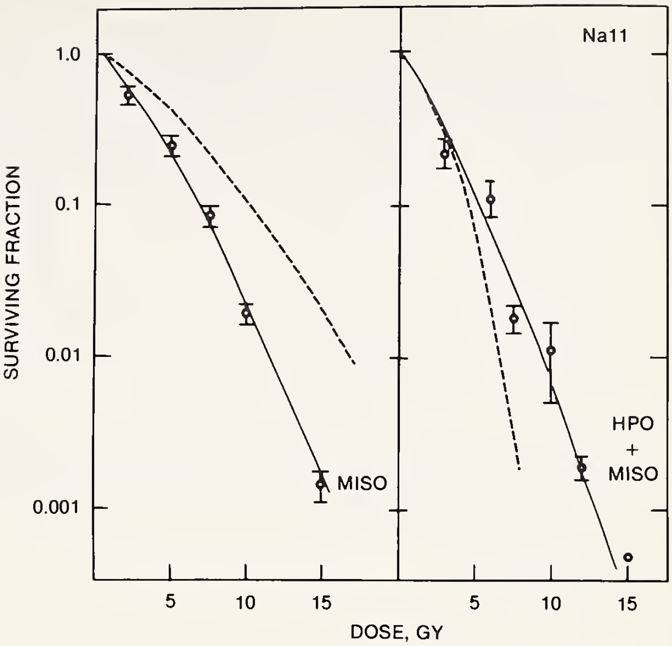


FIGURE 4.—Response of Na11 tumor cells irradiated in situ following injection of 1 mg of misonidazole/g or under HPO+misonidazole. *Left panel:* ——=misonidazole alone; — — —=control (saline injected). *Right panel:* ——=misonidazole+HPO; — — —=fully oxygenated cells in vitro.

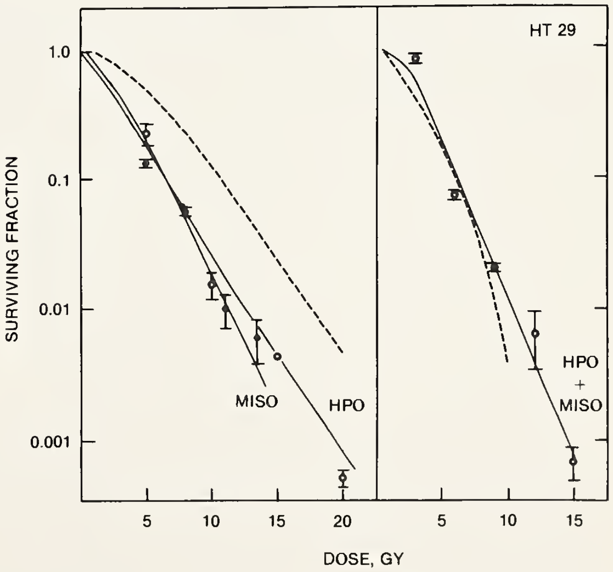


FIGURE 5.—Response of HT29 tumor cells irradiated in situ following injection of 1 mg of misonidazole/g and/or under HPO. *Left panel:* ——=misonidazole or HPO; — — —=control (saline injected). *Right panel:* ——=misonidazole+HPO; — — —=fully oxygenated cells in vitro.

TABLE 1.—Extent of sensitization

Treatment	Surviving fraction	Dose-modifying factor[a]		
		HRT18	NA11	HT29
Misonidazole	0.1	1.8	1.6	1.8
	0.01	1.7	1.5	1.5
HPO	0.1	1.4	—	1.7
	0.01	1.3	—	1.3
Misonidazole + HPO	0.1	2.0	1.8	1.7
	0.01	2.0	1.9	1.7

[a] Dose to reduce surviving fraction to given level/dose to reduce surviving fraction to same level in presence of sensitizer.

The extent of sensitization by misonidazole and/or HPO is summarized in table 1; the results are expressed in terms of the dose-modifying factor, which was calculated for surviving fractions of 0.1 and 0.01.

DISCUSSION

Among the mechanisms that have been invoked to explain the response of the human tumor xenografts irradiated in situ is the effect of intercellular contact (*1*). Hill et al. (*4*) reported such an effect for the KHT tumor, which was demonstrable as an enhancement of the shoulder of the survival curve for cells irradiated as small lung tumors or as single cells at short times after excision from the tumor. Our experiments using cells from human tumor xenografts failed to show any difference in radiosensitivity for cells irradiated at intervals after excision of the tumor, suggesting that intercellular contact was not influencing the results observed when these tumors were irradiated in situ. The results of these experiments also eliminate the possibility that radiation resistance is attributable to the age distribution of tumor cells in situ.

The radiation survival curves of many tumors in rodents (*5–7*) and humans (*8*) that were irradiated in situ have been interpreted as consisting of three components: a shoulder, an initial negative slope similar to that for aerated cells treated in vitro, and a terminal portion at high doses, which has the shallower slope characteristic of hypoxic cells. For the human tumor xenografts studied here, the survival curve is monophasic, with no steep initial portion. A similar type of curve has been reported for some rodent tumors, including the EMT-6 adenocarcinoma under certain conditions (*9,10*), and for a mouse osteosarcoma (*11*). The RIF-1 tumor implanted im also has a monophasic survival curve with a slope intermediate between that of well-oxygenated cells and that of fully hypoxic cells (*12*).

All three tumors contain a fraction of severely hypoxic cells, the size of which is indicated by the position of the terminal portion of the survival curves. The radiation sensitivity of the remaining tumor cells appears, from the shape of the survival curve, to be intermediate between that of completely aerated cells and that of severely hypoxic cells. A large proportion of this population is sensitized to radiation by either misonidazole or HPO, indicating that the radiation response is dependent on the level of oxygenation and that, presumably, at least part of this population consists of cells at intermediate levels of oxygenation. Cells made acutely hypoxic as a result of transient stoppages in blood flow may also contribute to the overall radiation response. For the HRT18 and Na11 tumors, misonidazole sensitized cells more effectively than HPO, and the effect of the two agents together was approximately additive (table 1). For the HT29 tumor, however, treatment with misonidazole, HPO, or misonidazole plus HPO produced a similar degree of sensitization. The finding that some apparently hypoxic cells were not sensitized by misonidazole may be explained on the basis of the observation of Blasberg et al. (*13*), who found that distribution of misonidazole to central tumor regions was limited in cases of low blood flow. The observation that radiosensitization by HPO was only partially effective is consistent with the presence of a fraction of acutely hypoxic cells resulting from transient interruptions in blood flow. Such cells would not be sensitized by procedures such as exposure to HPO, which act by increasing the distance that oxygen is able to diffuse away from blood vessels.

This is the first report of radiosensitization of tumor cells in situ by the combined use of HPO and misonidazole, and the results suggest that the procedure could be effective in targeting separate populations of cells refractory to radiotherapy. Presumably, partially hypoxic cells occur in human tumors under appropriate conditions, so that the proportion of the tumor cell population sensitized by hypoxic cell sensitizers will exceed the conventionally defined hypoxic fraction.

REFERENCES

(*1*) GUICHARD M, DERTINGER H, MALAISE EP: Radiosensitivity of four human tumor xenografts. Influence of hypoxic and cell to cell contact. Radiat Res 95:602–609, 1983.

(*2*) GUICHARD M, GOSSE C, MALAISE EP: Survival curve of human melanoma in nude mice. J Natl Cancer Inst 58:1665–1669, 1977.

(*3*) TOMPKINS WAF, WATRACH AM, SCHMALE JD, et al: Cultural and antigenic properties of newly established cell strains derived from adenocarcinomas of the human colon and rectum. J Natl Cancer Inst 52:1101–1110, 1974.

(*4*) HILL RP, NG R, WARREN BF, et al: The effect of intercellular contact on the radiation sensitivity of KHT sarcoma cells. Radiat Res 77:182–193, 1979.

(*5*) BARENDSEN GW, BROERSE JJ: Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV x-rays. II. Effects of fractionated treatments applied 5 times a week for several weeks. Eur J Cancer 6:89–109, 1970.

(*6*) ROCKWELL S, KALLMAN RF: Cellular radiosensitivity and tumor radiation response in the EMT-6 cell system. Radiat Res 53:281–294, 1973.

(*7*) SHIPLEY WU, STANLEY JA, STEELE GG: Tumor size dependency on the radiation response of the Lewis lung carcinoma. Cancer Res 35:2488–2493, 1975.

(*8*) COURTENAY VD, SMITH IE, PECKHAM MJ, et al: In vitro and in vivo radiosensitivity of human tumor cells obtained from a pancreatic tumor xenograft. Nature 263:771–772, 1976.

(*9*) RASEY JS, CARPENTER RE, NELSON NJ: Response of EMT-6 tumor to single fractions of x-rays and cyclotron neutrons. Evaluation and comparison of multiple end points. Radiat Res 71:430–446, 1977.

(*10*) FU K, PHILLIPS TL: The relative biological effectiveness (RBE) and oxygen enhancement ratio (OER) of neon ions for the EMT-6 tumor system. Radiology 120:439–441, 1976.

(*11*) van Putten LM, Lelieveld P, Broerse JJ: Response of a poorly oxygenating mouse osteosarcoma to x-rays and to fast neutrons. Eur J Cancer 7:153–160, 1971.

(*12*) Brown JM, Twentyman PR, Zamvil SS: Response of the RIF-1 tumor in vitro and in C3H/Km mice to x-radiation (cell survival, regrowth delay, and tumor control), chemotherapeutic agents, and activated macrophages. JNCI 64:605–611, 1980.

(*13*) Blasberg R, Horowitz M, Strong J, et al: Regional measurements of [^{14}C] misonidazole and blood flow in subcutaneous RT-9 experimental tumors. Cancer Res 45:1692–1701, 1985.

Glutathione as a Determinant of Cellular Response to Doxorubicin[1]

F. Y. F. Lee,* A. R. Vessey, and D. W. Siemann[2,3]

ABSTRACT—We have studied in detail the relationship between glutathione (GSH) depletion and sensitivity of HEp3 human carcinoma cells to doxorubicin [Adriamycin (ADR)]. Exponentially growing HEp3 cells were incubated with L-buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, for different periods so that a range of GSH depletion could be obtained. These GSH-depleted cells were then treated with a combination of BSO and ADR (1 μg/ml) for various durations. Under these conditions, the cytotoxicity of ADR was significantly enhanced by GSH depletion. The extent of ADR dose enhancement was found to be inversely proportional to cellular GSH level at the time of ADR treatment. Furthermore, it was shown that the dose-enhancement factors (DEF) also correlated with the duration of combined BSO and ADR treatment. For example, at a GSH level of 45% of untreated control, 18.5 ± 3 fmol/cell or $4.8 \pm 0.3 \times 10^{-3}$ fmol/μm^3 (±SD), DEF of 8.0, 6.4, and 5.0 were obtained for treatment periods of 3 hours, 2 hours, and 1 hour, respectively. Further study showed that the GSH kinetics differed significantly for the different treatment times, which indicates that GSH kinetics may be an important factor in determining the intrinsic sensitivity of HEp3 cells to ADR. Furthermore, the kinetics of GSH response to ADR varied significantly between cell lines. In the study of the effect of such differences, the GSH kinetics of 3 human ovarian tumor cell lines with different intrinsic sensitivities to ADR were investigated. It was found that, although cellular GSH concentrations bore little relation to ADR sensitivity, a better correlation existed between the area under the GSH concentration-time curve and sensitivity to ADR.—NCI Monogr 6:211–215, 1988.

Researchers have long thought GSH to be a major component of the cellular defense mechanism against toxic challenges such as ionizing radiation, cytotoxic drugs, and hyperthermia (*1*). The recent finding (*2–4*) that human tumor cells contain very high levels of GSH has led to the suggestion that it is an important factor limiting the therapeutic efficacy of conventional cancer treatment. However, the precise role of GSH has not been fully elucidated because of a lack of agents that specifically altered cellular GSH levels while being devoid of side effects. The synthesis of BSO, a specific inhibitor of GSH biosynthesis (*3,5*), has effectively overcome this difficulty. Enhancement by BSO of the cytotoxic effect of ionizing radiation (*3,6*) and some antineoplastic agents (*7,8*) have recently been reported.

Development of resistance to ADR, one of the most widely used chemotherapeutic agents, is a subject of intense current investigation. Early work with animal tumor cell systems has shown acquired resistance to ADR to be related to impaired influx and accumulation of the drug (*9–11*). Ling and co-workers (*12*) have also found alteration in drug transport in a Chinese hamster ovary cell line exhibiting pleiotropic drug resistance. Further reports have related overexpression of a plasma membrane glycoprotein to impaired drug transport and hence the multidrug resistance phenotype (*13*). However, no consensus has yet been reached regarding the mechanism of resistance that predominates in the clinical setting. Indeed, recent reports of studies on human tumor lines suggested that factors other than drug transport are responsible (*14,15*). Our present study focuses on the role of GSH in determining the sensitivity of human tumor cells to the cytotoxicity of ADR.

MATERIALS AND METHODS

Cell culture.—Derived from a metastatic buccal mucosa carcinoma (*16*), HEp3 cells were grown in F-12 nutrient mixture obtained from GIBCO (Grand Island, NY), supplemented with 15% FCS, 1 m*M* glutamine, and 10 m*M* HEPES. Three-day-old exponentially growing cells plated at 2×10^5 cells/75 cm^2 plastic culture flask [flasks produced by Corning Glass Works (Corning, NY)] were used for all experiments.

Ovarian tumor lines, derived from patients with epithelial ovarian cancer, were grown in alpha-minimum essential medium supplemented with 10% FCS, 5 m*M* glutamine, and 10 m*M* HEPES. The details of their establishment as tissue cultures and their pathology and treatment history are reported elsewhere (*2*).

Cytotoxic drug exposure.—The BSO, obtained from Chemical Dynamic Corp. (South Plainfield, NJ) was dissolved in PBS immediately before use. The ADR, obtained from Adria Laboratories Inc. (Columbus, OH), was dissolved in PBS, pH 7.4, at a concentration of 1 mg/ml and stored at −75° C for no longer than 4 weeks.

Cells in exponential growth in 75-cm^2 plastic flasks were washed once with Ca^{2+}- and Mg^{2+}-free PBS; and 0.01% trypsin from Cooperbiomedical (Malvern, PA) and 0.02% EDTA from Sigma Chemical Co. (St. Louis, MO) were

---

ABBREVIATIONS: GSH = glutathione; BSO = L-buthionine sulfoximine; ADR = doxorubicin (Adriamycin); HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCS = fetal calf serum; PBS = phosphate buffer solution; HPLC = high-performance liquid chromatography; DEF = dose-enhancement factor(s); AUC = area under the concentration-time curve.

---

[1] Supported by Public Health Service grants CA-11051, CA-38637, and CA-36858 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Experimental Therapeutics Division and Department of Radiation Oncology, University of Rochester Cancer Center, NY.

[3] We thank Susan Curnick, Amy Flaherty, and Brenda King for technical assistance and Barbara Granger for preparing the manuscript.

* *Reprint requests:* F. Y. F. Lee, Ph.D., Experimental Therapeutics Division, University of Rochester Cancer Center, 601 Elmwood Ave., Box 704, Rochester, NY 14642.

added. Single cells obtained were suspended at a concentration of 2.5×10^5/ml in complete media plus FCS in a type I vial at 37° C, as described by Whillans and Rauth (*17*). Cells, continuously gassed with a 95% air and 5% CO$_2$ mixture during the drug incubation period, were then allowed to equilibrate in this condition for 30 minutes before drug exposure.

For ADR treatments, 10 μl of a stock solution of ADR was given per milliliter of cells. For experiments in which cells were exposed to BSO before ADR, BSO was given at various times prior to ADR treatment so that a range of GSH concentrations at the time of cytotoxic challenge could be obtained. At the end of drug exposure, a 1-ml aliquot of cells was washed by suspension in 14 ml cold medium; then it was centrifuged at 1,000 *g* for 10 minutes at 4° C. The cell pellet was resuspended in complete medium plus FCS, the cells counted with a hemacytometer, and survival determined by the plating of various dilutions in cell culture dishes. Twelve days later, the dishes were stained with crystal violet, and the number of colonies containing more than 50 cells were counted for determination of cell survival.

Sample preparation for high-performance liquid chromatography.—At various times during drug exposure, aliquots of cells were removed for HPLC analysis. Cells were homogenized with 200 μl of 20 m*M* 5-sulfosalicylic acid, also from Sigma Chemical Co. The homogenates were centrifuged for 40 seconds (Eppendorf Microcentrifuge). The GSH in the supernatant is derivatized by the fluorescent reagent monobromobimane [3-(bromoethyl)-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazole-1,7-dione)8-dione]. Aliquots (180μl) of the supernatant were pipetted into 3-ml glass tubes containing 12 μl *N*-ethylmorpholine (0.5 *M* in 2.0 m*M* potassium hydroxide) and 2 μl monobromobimane (50 m*M* in acetonitrile). The sample is immediately vortexed and stored in the dark at 4° C before analysis.

High-performance liquid chromatography.—Concentrations of GSH and ADR were assayed by pair-ion HPLC. The HPLC equipment, from Waters Associates (Milford, MA), consisted of model 6000A chromatography pumps, model 710B automated sample processors (WISP), Data Module, model 720 system controller, Z-module, and model 420 fluorescence detector.

Separation of GSH was performed on Waters Radial-PAK reverse-phase, bonded octadecylsilane (C18) cartridge columns (8 mm inner diameter, 5 μm diameter spherical particles). The mobile phase consisted of 23% acetonitrile in 40 m*M* ammonium phosphate buffer, pH 7.2, containing 5 m*M* *tetra*-butylammonium hydroxide. The effluent was monitored for fluorescence with 340 nm excitation and emission at greater than 410 nm.

Cell sizing.—Cells were suspended in PBS at a concentration of $2\text{–}4 \times 10^4$ cells/ml, and cell volume was determined by a model ZBI Coulter Counter equipped with a model C1000 Channelyzer from Coulter Corp. (Hialeah, FL).

RESULTS

Effects of L-Buthionine Sulfoximine on Cellular Glutathione Content and Survival

Initial experiments were performed in HEp3 cells so that a dose of BSO could be selected that produces optimum rate of GSH depletion with minimal toxicity. Figure 1 shows the extent of GSH depletion 6 hours following various concentrations of BSO. It can be seen that GSH depletion increased with increasing BSO concentration from 0.01–1.0 m*M*, but it leveled off thereafter. Therefore, a concentration of 1 m*M* BSO was chosen for the following series of experiments.

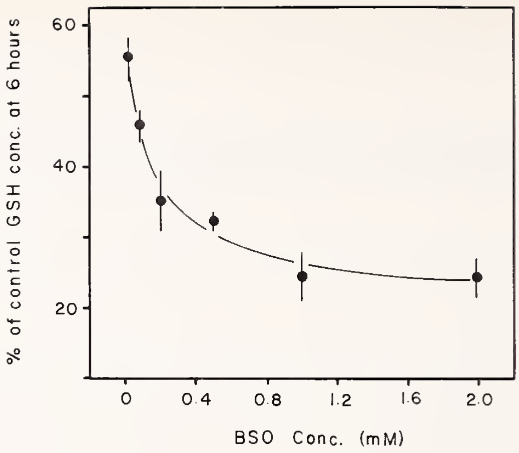


FIGURE 1.—Concentration-response of BSO on GSH depletion in HEp3 cells after a 6-hr incubation with BSO. Data are from 2 independent experiments. *Error bars* indicate ± 1 SD ($n=6$). Conc. = concentration.

The complete kinetics of GSH depletion by 1 m*M* BSO and the corresponding effects on cell survival of HEp3 are shown in figure 2. The rate of depletion appeared linear for

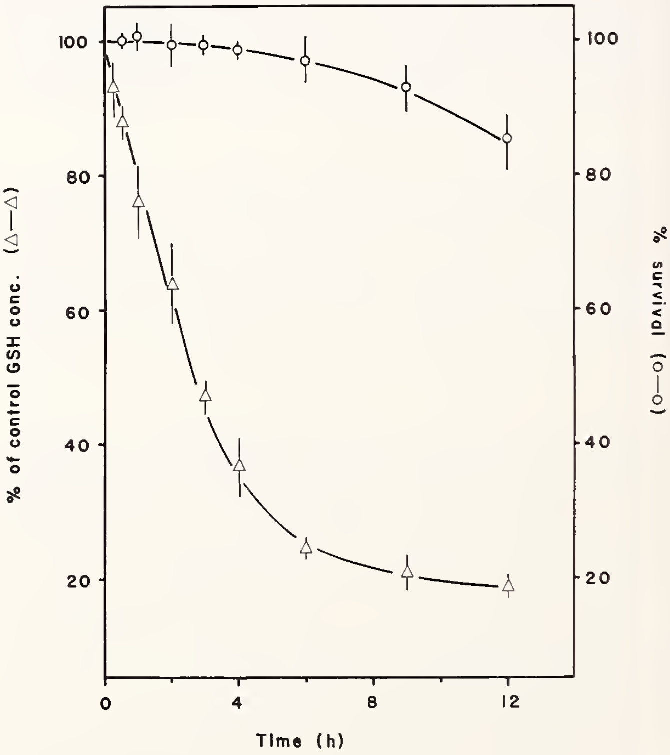


FIGURE 2.—Effects of treatment time with 1 m*M* BSO on GSH concentration and on cell survival. Results are from 2 independent experiments. *Error bars* represent ± 1 SD (n = 6–9).

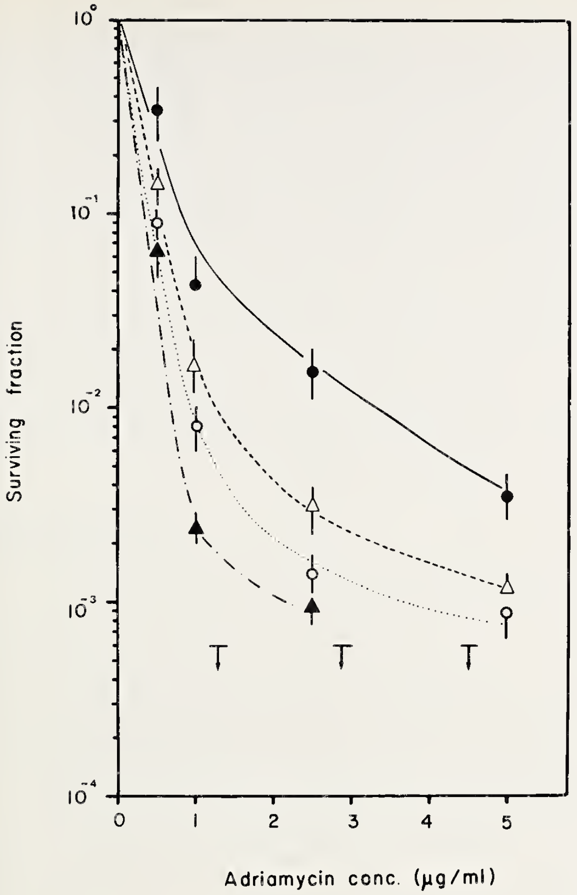


FIGURE 3.—Effects of pretreatment with 1 m*M* BSO for different times on the clonogenic survival of HEp3 cells following a 1-hr incubation with 1 µg ADR/ml. ● = no BSO pretreatment; △ = 1 hr, ○ = 2 hr, and ▲ = 3 hr BSO pretreatment. *Error bars* represent ± 1 SD. Data are from a typical experiment. Two repeat experiments gave similar results.

approximately 3 hours and followed first-order kinetics up to 6 hours but slowed significantly thereafter. Cell survival was not significantly reduced after 6 hours of exposure (survival = 98%), but increased cytotoxicity was seen with more prolonged treatment time. Exposure in the present studies never exceeded 6 hours.

Effects of Glutathione Depletion by L-Buthionine Sulfoximine on Doxorubicin Cytotoxicity

The cytotoxicity of ADR was enhanced by BSO. The results of a typical experiment in which HEp3 cells were pretreated with 1 m*M* BSO for 1 hour, 2, or 3 hours prior to a range of ADR doses administered for 1 hour are given in figure 3. For untreated controls, the GSH content was 18.5 ± 3 fmol/cell or $4.8 \pm 0.3 \times 10^{-3}$ fmol/µm^3 (±SD). At the time of ADR treatment, cellular GSH concentrations were 100%, 75%, 60%, and 46% of untreated control for the 0-, 1-, 2-, or 3-hour BSO pretreatments, respectively. The extent to which the cytotoxicity of ADR was enhanced by BSO pretreatment was calculated from data such as those shown in figure 3 and found to be proportional to the cellular GSH concentrations at the time of ADR treatment (fig. 4). The results indicate that the lower the GSH concentration at the time of ADR treatment, the higher the DEF.

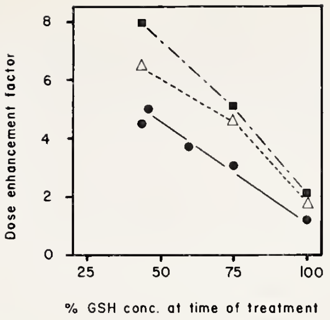


FIGURE 4.—Relationship between GSH concentration at the time of ADR treatment and the DEF of ADR cytotoxicity. ● = 1-hr, △ = 2-hr, and ■ = 3-hr combined treatment with 1 m*M* BSO and 1 µg ADR/ml. Results are calculated from 3 independent experiments. DEF = isoeffect concentration for ADR alone/isoeffect concentration for ADR and BSO, when the isoeffect was 2-log cell lethality.

Furthermore, DEF also increased with increasing ADR treatment time (fig. 4).

Effects of Doxorubicin Treatment on Cellular Glutathione Contents and the Influence of L-Buthionine Sulfoximine

Upon treatment with ADR, the GSH contents of HEp3 cells decreased to a nadir at 30–60 minutes, subsequently recovered, and then overshot to levels above the initial concentration (fig. 5). With BSO treatment, no recovery was evident. This was independent of whether the BSO treatment was given simultaneously 1 hour or 3 hours before ADR (fig. 5). With simultaneous treatment, the GSH kinetics of the treated cells for the first hour closely resembled those of cells not exposed to BSO but diverged dramatically thereafter. For the 1- and 3-hour BSO pretreatment, initial GSH levels at the time of treatment were lower, but

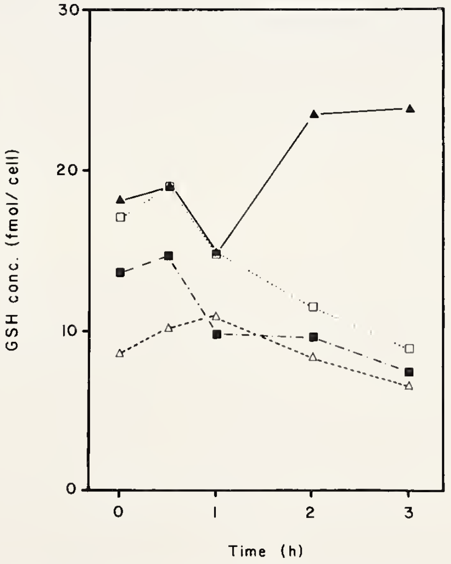


FIGURE 5.—Effects of ADR administered at zero time on the GSH kinetics of HEp3 cells treated with 1 m*M* BSO (△) 3 hr before ADR; (■) 1 hr before, (□) simultaneously with ADR, or (▲) with no BSO treatment. *Each datum point* is the mean of 4–6 determinations. Results are from 2 independent experiments.

TABLE 1.—Relationship between cellular GSH content, GSH AUC, and clonogenic cell survival following treatment with ADR[a]

Cell line	Surviving fraction[b]	GSH AUC[c]	GSH concentration[b,d]
PEA	$6.5 \pm 1.7 \times 10^{-3}$	1.05	5.55 ± 1.2
SAU	$9.5 \pm 4.2 \times 10^{-3}$	1.26	7.74 ± 1.8
SKA	$3.1 \pm 1.1 \times 10^{-2}$	1.67	5.66 ± 0.9

[a] Cells were treated with 1 μg ADR/ml for 3 hr.

[b] Values are expressed as mean ± 1 SD.

[c] Values are expressed in femtomoles · minute^{-1} · micrometer^{-3} from time zero to 3 hr and were estimated by Simpson's rule.

[d] Values are expressed in attomoles per cubic micrometer.

the shape of the GSH kinetic curves were generally similar (fig. 5).

Influence of Glutathione Content Changes on Cellular Sensitivity to Doxorubicin

The above results suggested that at least two factors may have bearing on ADR cytotoxicity. As expected, the extent of GSH depletion before ADR treatment was proportional to the resultant DEF (fig. 4). However, in addition, the increased DEF observed with increasing treatment time when both ADR and BSO were present simultaneously suggested that changes in GSH content during treatment (fig. 5) should also be considered. A parameter which might satisfy these requirements is the area under the GSH AUC. Therefore, we performed preliminary studies on both clonogenic cell survival and GSH content in 3 ovarian tumor lines established directly from patients with epithelial tumors to see if GSH AUC would correlate with cell survival. The cell lines investigated demonstrated widely different intrinsic sensitivities to ADR. For the present set of experiments, the AUC from 0 to 3 hours ($AUC_{0\text{-}3\,hr}$) was used. The 3-hour period was chosen because during this time ADR still influenced GSH kinetics; the intracellular free ADR accumulated during a 1-hour exposure required a 2-hour postexposure period to be dissipated to 5%–15%. The initial GSH concentration, GSH $AUC_{0\text{-}3\,hr}$ and cell survival values for the 3 ovarian lines are given in table 1. The data suggest that GSH $AUC_{0\text{-}3\,hr}$ offers a better correlation with cell survival than does initial cellular GSH concentration.

DISCUSSION

The findings of our present study show the importance of GSH in determining the responsiveness of tumor cells to ADR. The depletion of GSH by BSO in the human tumor lines HEp3 was rapid and dose dependent. Maximum depletion of GSH was achieved with BSO concentration between 0.5 and 1.0 m*M* (fig. 1). The potency of BSO is well illustrated by GSH depletion to 56% of control at 6 hours with a BSO dose as low as 10 μ*M* (fig. 1). In a comparison of the present results with those of earlier cell-free studies with purified γ-glutamylcysteine synthetase, it was noted that in the latter studies, 1 μ*M* BSO inhibited the enzyme by 52%, and complete inhibition was achieved with 20 μ*M* of BSO (*5*). The difference in the concentration of BSO required for complete enzyme inhibition and maximum depletion of cellular GSH may be due to inadequate drug uptake into the cell and/or access barrier to BSO for the enzyme. Furthermore, because under physiologic conditions GSH maximally inhibits γ-glutamylcysteine synthetase by nonallosterically binding to the enzyme (*18*), there may be conformational differences that make the in situ enzyme less susceptible to inhibition by BSO than is the purified enzyme. Nevertheless, it is clear that potent inhibition of GSH synthesis can be achieved with micromolar concentrations of BSO, a concentration that is readily achievable with nontoxic doses of BSO in mice (Lee FYF, Siemann DW, unpublished results).

The present results show that in HEp3 cells toxicity due to BSO can be avoided if the exposure time does not exceed 6–8 hours (fig. 2). Prolongation of exposure, however, does produce reduction in cell survival (fig. 2). This observation is consistent with the finding of Dethlefsen et al. (*19*) that extended GSH depletion by BSO produced a delay in cell-cycle progression and cell death.

It has now been well established that GSH depletion can lead to enhanced cytotoxicity of a number of anticancer agents (*7,8*). In the present studies, the cellular GSH levels immediately before and during ADR treatment were monitored, and the relationship between the extent of GSH depletion and augmentation of cytotoxicity was determined. The results showed that the DEF for ADR increased with decreasing initial GSH concentration, which is strongly supportive of the idea that GSH plays an important role in determining cellular sensitivity to ADR.

In combination with selected chemotherapeutic agents, BSO is likely to be undergoing clinical trials in the near future. However, information that may assist the rational selection of either drug combination or treatment schedule is relatively sparse. Because cardiac toxicity is dose limiting for ADR in man and BSO has been shown to deplete cardiac GSH content severely and chronically in mice (*20*), the use of BSO plus ADR in patients is potentially dangerous. Nonetheless, this study with combined ADR and BSO may provide some general information that may help in the selection and optimal usage of other cytotoxic drugs in combination with BSO. The finding that DEF for ADR increased with increasing treatment time when both BSO and ADR were simultaneously present (fig. 4) suggests two conditions that may improve the effectiveness of a combination drug regimen. First, effective BSO concentration should be maintained for a time after the cytotoxic agent is administered. Second, a cytotoxic agent with a relatively long biologic half-life may be advantageous. Studies of GSH kinetic changes show that when both BSO and ADR were present, cellular GSH concentrations continuously declined (fig. 5). In contrast, with ADR alone, cellular GSH concentration recovered rapidly after an initial decline, even to levels considerably above steady state (fig. 5). This difference in GSH kinetics may provide an explanation for the correlation between DEF and treatment time with combined BSO and ADR (fig. 4).

The GSH kinetics data (fig. 5) and those for cell survival (fig. 4) suggest two GSH parameters that can influence cellular sensitivity to ADR; 1) GSH concentration at the time of cytotoxic challenge, and 2) GSH kinetic changes *during* cytotoxic challenge. In an attempt to incorporate these two parameters, we have utilized the GSH AUC concept. A most interesting question regarding the clinical relevance of GSH levels (either inherent or following manipulation) is whether differences in GSH recovery kinetics per se in different human tumor cells could correlate with their intrinsic sensitivities to chemotherapy. To address this question, we have conducted preliminary studies with 3 human ovarian tumor cell lines (table 1). The results indicate an apparent correlation between GSH AUC and the degree of resistance to ADR. In contrast, little correlation appears to exist between GSH concentration and drug sensitivity. To evaluate further the generality of this concept, we currently are studying a panel of human tumor cell lines with differing sensitivity to ADR.

In conclusion, the present results have shown that GSH plays a vital role in determining the extent of cellular damage by ADR. Manipulation of GSH by the thiol-depleting agent BSO significantly potentiates the efficacy of ADR, but the extent of potentiation is dependent on the intrinsic cellular GSH level and GSH recovery kinetics.

REFERENCES

(*1*) Arrick BA, Nathan CF: Metabolism as a determinant of therapeutic efficacy: A review. Cancer Res 44:4224–4232, 1984.

(*2*) Lee FYF, Vessey AR, Siemann DW, et al: Kinetic changes in cellular glutathione concentration during cytotoxic challenge as a determinant of Adriamycin cytotoxicity. Cancer Res. In press.

(*3*) Biaglow JE, Clark EP, Epp ER, et al: Nonprotein thiols and the radiation response of A549 human lung carcinoma cells. Int J Radiat Biol 44:489, 1983.

(*4*) Mitchell JB, Morstyn G, Russo A, et al: In vitro radiobiology of human lung cancer. Cancer Treat Symp 2:3–10, 1985.

(*5*) Griffith OW, Meister A: Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine. J Biol Chem 254:7558–7560, 1979.

(*6*) Shrieve DC, Denekamp J, Minchinton AI: Effects of glutathione depletion by buthionine sulfoximine on radiosensitization by oxygen and misonidazole in vitro. Radiat Res 102:283–284, 1985.

(*7*) Hamilton TC, Winker MA, Louie KG, et al: Augmentation of Adriamycin, melphalan and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. Biochem Pharmacol 34:2583–2586, 1985.

(*8*) Ozols RF: Pharmacological reversal of drug resistance in ovarian cancer. Semin Oncol 7(suppl 4):7–11, 1985.

(*9*) Dano K: Outward transport of daunorubicin in resistant Ehrlich ascites tumor cells. Biochim Biophys Acta 323:466–483, 1973.

(*10*) Inaba M, Johnson RK: Uptake and retention of Adriamycin and daunorubicin by sensitive and anthracycline resistant sublines of P388 leukemia. Biochem Pharmacol 27:2123–2130, 1978.

(*11*) Sutherland RM, Eddy HA, Bareham B, et al: Resistance to Adriamycin in multicellular spheroids. Int J Radiat Oncol Biol Phys 5:1225–1230, 1979.

(*12*) Ling V, Kartner N, Sudo T, et al: Multidrug-resistance phenotype in Chinese hamster ovary cells. Cancer Treat Rep 67:869–874, 1983.

(*13*) Kartner N, Riordan JR, Ling V: Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science 221:1285–1288, 1983.

(*14*) Louie KG, Hamilton TC, Winker MA, et al: Adriamycin accumulation and metabolism in Adriamycin-sensitive and resistant human ovarian cancer lines. Biochem Pharmacol 35:467–472, 1986.

(*15*) Harker WG, Bauer D, Etiz BB, et al: Verapamil-mediated sensitization of doxorubicin-selected pleiotropic resistance in human sarcoma cells: Selectivity for drugs which produce DNA scission. Cancer Res 46:2369–2373, 1986.

(*16*) Toolan HW: Transplantable human neoplasms maintained in cortisone-treated laboratory animals H.S#1; H.Ep#1; H.Ep#2; H.Ep#3; and H.Emb.Rb#1. Cancer Res 14: 660–666, 1954.

(*17*) Whillans DW, Rauth AM: An experimental and analytical study of oxygen depletion in stirred cell suspensions. Radiat Res 84:97–114, 1980.

(*18*) Meister A, Anderson ME: Glutathione. Annu Rev Biochem 52:711–760, 1983.

(*19*) Dethlefsen LA, Biaglow JE, Peck VM, et al: Toxic effects of extended glutathione depletion by buthionine sulfoximine on murine mammary carcinoma cells. Int J Radiat Oncol Biol Phys 12:1157–1160, 1986.

(*20*) Lee FYF, Allalunis-Turner MJ, Siemann DW: Depletion of tumour *versus* normal tissue glutathione by buthionine sulfoximine. Br J Cancer 56:33–38, 1987.

Cell-cycle Dependence of X-ray Oxygen Effect: Role of Endogenous Glutathione [1]

E. A. Blakely,[2,*] R. J. Roots,[2] P. Y. Chang,[2] L. Lommel,[2] L. M. Craise,[2] E. H. Goodwin,[2] E. Yee,[2,3] D. P. Dodgen,[4] and W. F. Blakely[4]

ABSTRACT—**The oxygen effect was measured in human T-1 cell populations synchronized by mitotic selection and x-irradiated in vitro after they were allowed to progress to six different ages during the division cycle. Survival curves and dose-ratio calculations with 95% confidence intervals were obtained from computer fits of the data to the linear-quadratic model. The oxygen enhancement ratio (OER) values at the 1% survival level were 2.6 ± 0.08 in G_1/early S phase and increased to 3.0 ± 0.15 in late S/G_2 phase. The OER values at 10% survival increased linearly from 2.6 ± 0.2 for G_1-phase cells to 3.2 ± 0.2 for late S/G_2-phase cells. The increased OER in S-phase cells was the result of a greater hypoxic radioresistance compared with that measured with G_1-phase cells. In parallel experiments with synchronized cell populations, glutathione (GSH) and glutathione disulfide levels were measured by the Tietze assay and also were found to increase over the same period. The molecular mechanisms responsible for the radiation response involve a number of factors, one of which in this cell line may be GSH levels, especially under conditions of hypoxic exposure. Our data are consistent with the hypothesis that G_1- to late S-phase, age-dependent fluctuations in GSH content may be correlated with changes in OER during the human T-1 cell cycle. Changes in GSH content relative to its constitutive levels in the cell and alternative reductive factors (i.e., protein thiols), as well as their cellular location, may be important factors in the comparison of these findings to other cell lines.—NCI Monogr 6:217–223, 1988.**

One of the early descriptions of the OER phenomenon in mammalian cells was made by Gray (*1*). Subsequently, the response to low linear energy transfer radiation during the cell division cycle was investigated for several cell lines under aerobic conditions (*2-6*). Few researchers (*7-13*) have compared the radiosensitivity of hypoxic to aerobic cells in different phases of the cell generation cycle. However, these investigations have in several cases suffered from poor experimental hypoxia, possible perturbations caused by drugs or agents used to induce synchrony or lack of a full cell-cycle analysis. In general, after sparsely ionizing (low linear energy transfer) irradiation under aerobic conditions, mammalian cells at the G_1 and the G_2/M positions in the generation cycle are more radiosensitive than are cells positioned in other phases, the most resistant phase being the late S phase. At reasonably low doses, i.e., a dose required to reduce survival to 0.37 or one required to reduce survival e^{-1} along the exponential portion of a survival curve for asynchronously growing cells, these differences are fairly small.

When the sensitivity of hypoxically irradiated cells has been compared with aerobically maintained cells for evaluation of the OER throughout the generation cycle (*7-13*), the conclusion commonly has been that the OER varied little if any during the cell generation cycle (*8-11*), although a lower OER in mitotic and early G_1-phase cells compared with the later S- and G_2-phase stages has been reported (*7,12,13*). In the latter cases, perturbations by methotrexate (*7*), a drug cytocidal to S-phase cells and therefore causing a partial synchrony, or a prolonged exposure to hypoxia (*12*) may have caused DNA perturbations and abnormal cell progression. Because we do not have available in the literature a complete study of the dependence or independence of the OER on cell age, we have measured OER values from an early G_1 stage into the following mitosis. In these experiments, OER magnitudes were measured throughout the generation cycle of human T-1 cells synchronized by mitotic selection. We observed statistically significant differences between the OER of G_1 cells and those measured for cells in late S/G_2 phase. These OER measurements vary in two ways, with survival level as has also been shown by others [e.g., *see* (*14,15*)], and secondly, with cell age, a phenomenon which hitherto has remained unresolved. In addition, preliminary measurements indicated that the intracellular total GSH levels may be related to the OER changes in the cell cycle.

MATERIALS AND METHODS

Cell line, culture conditions, and cell synchrony.—The human T-1 cell line and culturing conditions have been described in detail elsewhere (*16*). Mitotic T-1 cells were selected without drugs from an exponentially growing population with an automatic Talandic cell synchronizer as reported in (*17*). Cell volume distributions and autoradiography of [^{3}H]dThd-pulsed samples were completed for each experiment reported.

ABBREVIATIONS: OER = oxygen enhancement ratio(s); GSH = glutathione; NPSH = nonprotein sulfhydryl.

[1] Supported by Public Health Service grant CA-15184 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services; grant DE AC03-76SF00098 from the Department of Energy; and funding from the Defense Nuclear Agency Work Unit B5103.

[2] Biology and Medicine Division, Lawrence Berkeley Laboratory, University of California, Berkeley.

[3] Student Employment and Training Program, University of California, Riverside.

[4] Radiation Sciences Division, Armed Forces Radiobiological Research Institute, Bethesda, MD.

[*] *Reprint requests:* E. A. Blakely, Ph.D., Lawrence Berkeley Laboratory, 1 Cyclotron Rd., Bldg. 10, Rm. 209, Berkeley, CA 94720.

Irradiation methods.—Mitotically harvested cells were plated into custom-made 35-mm diameter glass dishes at about 2×10^4 cells/cm^2. At the appropriate cell age, the dishes were loaded into specially designed aluminum gassing chambers, and the cells were covered with 0.2 ml culture medium and then gassed for 20 minutes (*16*). Humidified nitrogen gas with 5% CO_2 and humidified air plus 5% CO_2 were used. A slight positive gas pressure was maintained during irradiation.

X-Irradiation of the cells was performed with a Philips RT250 x-ray unit operated at 225 kilovolt peak and 15 milliamperes at a dose rate of about 5.5 Gy/minute. The doses reported were not corrected for the dose back scattered from the glass. The exposures for the complete dose–response curves were planned so that the reported age of the cells at the time of irradiation represented the mean age of cultures exposed to generate the whole survival curve. After irradiation, the cells were covered with fresh culture medium and processed for clonogenic assays in a routine manner (*16*).

Three experiments were completed in which cellular survival was measured at 0.50- to 1.25-hour intervals covering the G_1 phase, through S phase, and into the first G_2/M border postmitotic harvest. All aerobically irradiated samples received an absorbed dose of 3.5 Gy, and hypoxically irradiated cells received an absorbed dose of 9.8 Gy, a 2.8-fold larger dose, which is approximately the OER value obtained at 10% survival for exponentially growing T-1 cells irradiated under these conditions (*16*). In another set of experiments, complete dose–response curves were generated for both air- and nitrogen-gassed samples at 3, 5, 8.1, 11, 14, and 17 hours after mitotic selection.

Autoradiography.—In each experiment, cell progression was monitored by incorporation of [^{3}H]dThd as described in (*17*). The autoradiography samples were held at room temperature for 20 minutes prior to labeling to coincide with the gas exchange period. The cooling of the cells to room temperature slowed the progression slightly, as compared with the kinetics of untreated cell populations we reported previously (*17*). From the data presented in figure 1, the duration of the phases of the cell cycle for the experiments reported here were calculated to be 6.5 hours (G_1 plus M), 9 hours (S phase), 4 hours (G_2 plus 1/2 M), and a total generation time of 19.5 hours. More than approximately 95% of the cells incorporated [^{3}H]dThd 11 hours after synchronization, an indication of reproducibly high-quality synchrony.

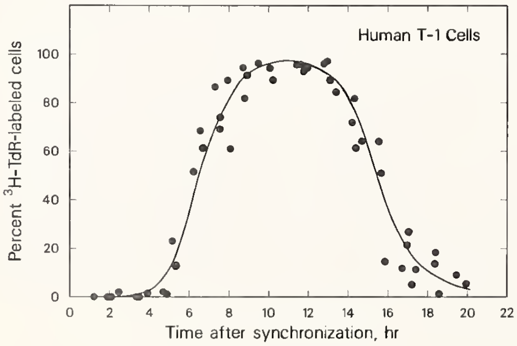


FIGURE 1.—Reproducibility of DNA synthetic phase in synchronized controls. Composite of DNA labeling patterns from autoradiographs of unirradiated controls pooled from 4 experiments pulsed with [^{3}H]TdR (dThd) at various times after synchronization. These control cultures were held at room temperature for a 20-min period prior to labeling to correspond to the gas exchange time in the irradiated samples. Analysis of these data indicated that under the conditions of the experiments reported here, the duration of the cell-cycle phases were M=0.7 hr, G_1=6.2 hr, S=9 hr, and G_2=3.7 hr.

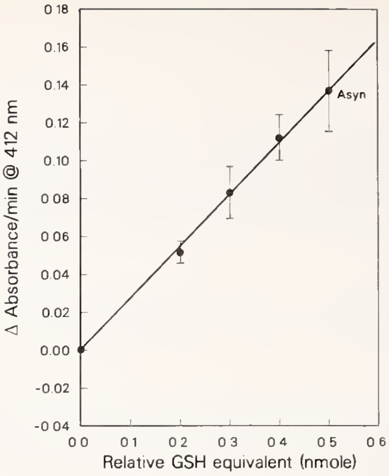


FIGURE 2.—A GSH-equivalent standard curve. Levels of cellular GSH and glutathione disulfide were measured by the Tietze assay with the internal standard technique on acid-extracted and heat-denatured cell extract supernatants from each cell population. Example standard curve shown here is for asynchronous cell populations. Linear regression best fits of the change in absorbance at 412 nm/min vs. relative GSH-equivalents (in nanomoles) was 0.78±0.02.

Glutathione measurements.—Both GSH and glutathione disulfide levels, expressed as GSH equivalents, were measured by the Tietze assay (*18*). Sample extracts were prepared by the addition of 0.5 ml of 0.6% 5-sulfosalicylic acid at 4° C to cell pellets which were then stored at −70° C until subsequent analysis. We found that γ-glutamyl transpeptidase, an enzyme capable of degrading GSH, was completely inhibited by a brief exposure to 0.6% of the acid (data not shown). At the time of GSH analysis, the 5-sulfosalicylic acid cell extract was rapidly thawed and incubated at 56° C for 20 minutes to facilitate protein denaturation and then was chilled to 4° C. Following centrifugation, the supernatant was removed and maintained at 4° C until measurements of GSH levels were completed, usually within 24 hours. An internal calibration standard (GSH) was used in the GSH measurements. Figure 2 presents the standard curve for the thiol measurements with asynchronous T-1 cells. A standard curve was generated for cells at each stage of the cell cycle studied. Ten to 20 aliquots were assayed for GSH from the samples at each time point.

Protein measurements.—Total cell protein was determined according to the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) which is based on the binding of Coomassie brilliant blue to protein (*19*). Aliquots of cells were lysed by sonication, and bovine serum albumin was used as the protein standard. Three or four replicates were measured for each sample.

Data analysis.—The intracellular contents of GSH and glutathione disulfide are expressed as GSH equivalents per total cell protein (nanomoles GSH/milligram protein). An

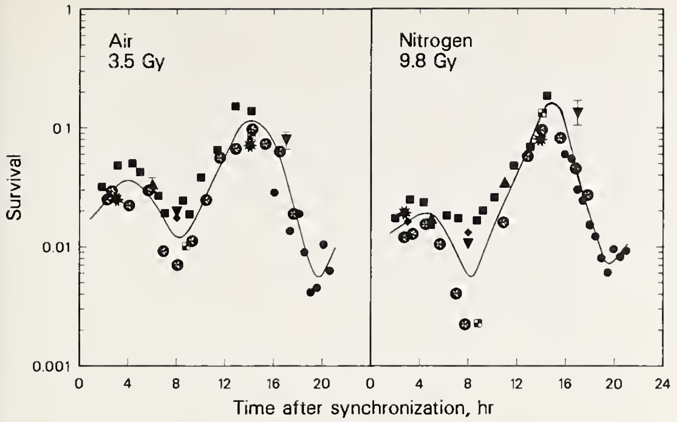


FIGURE 3.—X-Ray age response for exposures in air and in nitrogen. Survival data are pooled from 8 experiments, 5 of which represent survival measurements from complete dose–response curves at the 3.5- and 9.8-Gy dose levels for air- and nitrogen-saturated cells, respectively. Other three sets of data represent multiple survival measurements from cultures at various cell ages x-irradiated with 3.5 Gy in air or 9.8 Gy in nitrogen on glass petri dishes. Curves are best eye fits.

SE was estimated for each value based on the variances associated with GSH and cell protein measurements as described by Cleland (*20*).

Survival curves, dose–ratio calculations of OER, and associated 95% confidence intervals were obtained from computer fits of the data to the linear–quadratic model with programs developed by Albright (*21*).

RESULTS

Aerobic and Hypoxic Age Response Single Doses

Our initial experiments to examine the cell-cycle dependence of the oxygen effect were completed by synchronizing cells by mitotic selection, allowing them to progress to various ages, and then irradiating them with a fixed single dose under aerobic or hypoxic conditions. The survival data from 3 experiments of this type are plotted in figure 3. This figure also summarizes the survival measurements at the appropriate dose level taken from 5 experiments in which complete dose–response curves were generated. A curve has been drawn by eye through the compiled data for each gas condition of exposure with arithmetic means of the data.

There is some scatter in the data summarized from the 8 experiments, especially at the G_1/S phase interface (i.e., 5–8 hr after synchronization) under hypoxia; however, qualitatively, a trend in the data of figure 3 can be noted. Figure 4 permits an examination of the superimposed mean curves from figure 3. Perfect superimposition of the curves would indicate an OER of 2.8 at 10% survival at all ages for the x-ray dose studies. A comparison of the curves in figure 4 shows qualitatively that lower OER values are observed in the early part of the cell cycle and higher OER values later in S phase. It should be noted that the pooled data shown in figure 3 represent results from 8 experiments performed over a period of about 2 years.

To evaluate this observation in a more quantitative fashion, we constructed complete aerobic and hypoxic survival estimates from the data of figure 3. At the 3.4-Gy aerobic dose level, cellular radioresistance decreased as the cells aged from mid-G_1 to late S phase. This trend also occurred under hypoxic irradiation, but the radioresistance decreased more at the G_1/S interface and increased more in the middle to late S phase (data not shown).

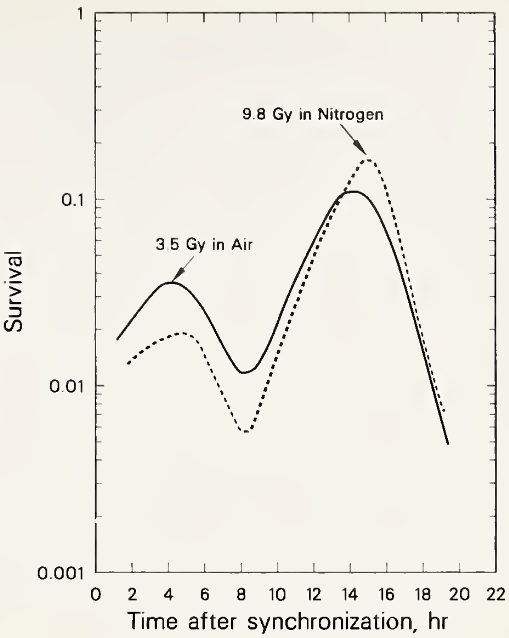


FIGURE 4.—Superimposed plots of best air and nitrogen fits of the data from fig. 3. Perfect superimposition of the curves would indicate an OER at 10% survival of 2.8 at all ages. Comparison shows qualitatively that lower OER values are observed in the early part of the cell cycle and higher OER values later in S phase.

Aerobic and Hypoxic Age Response Complete Dose Curves

Based on the data described above, a series of 5 experiments was completed under the conditions in which cells were mitotically selected, allowed to progress to various ages, and then irradiated with graded doses under aerobic

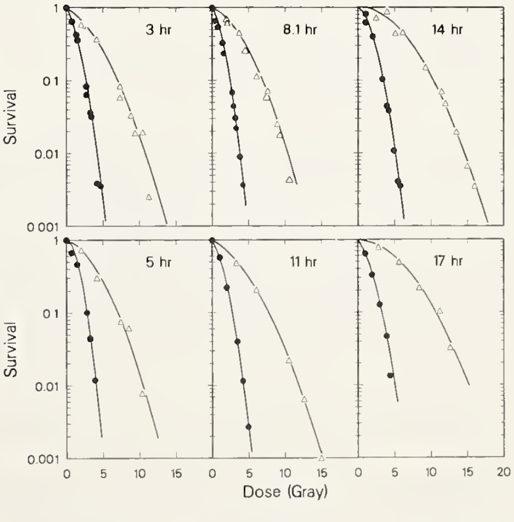


FIGURE 5.—Complete x-ray dose–response curves for cell populations irradiated postsynchronization in air (●) or in nitrogen (△) at 3, 8.1, or 14 hr (*upper panel*) or at 5, 11, or 17 hr (*lower panel*).

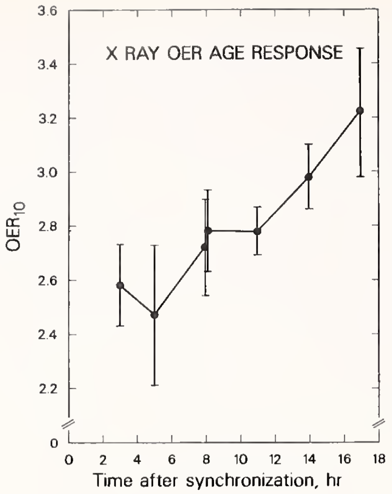


FIGURE 6.—Summary plot of computer-fitted OER values at 10% survival from complete aerobic and hypoxic dose-response curves measured with synchronized populations at six cell ages. Data are from 5 experiments.

and hypoxic conditions. The data from cell populations positioned at six phases in the cell cycle between 3 and 17 hours after synchronization are summarized in figure 5. The curves drawn through the data points are computer-assisted, best-fit regressions. Replicate experiments were completed for cell populations at 3, 8.1, and 14 hours. Only single experiments were completed at 5, 11, and 17 hours.

Dose-ratio calculations of OER values were made for the pair of curves at each cell age and are presented in table 1. At high doses (e.g., 1% survival level), the OER values increase from approximately 2.6±0.1 in G_1/early S phase to 3.0±0.2 in late S/G_2 phase. At lower doses (e.g., 10% survival level), the same trend is observed with the OER values again increasing from approximately 2.6±0.2 in G_1-phase cells to 3.2±0.2 in late S/G_2-phase cells. These results are also plotted in figure 6. An approximately linear increase in the OER is noted as the cells progressed from G_1 to G_2 phase.

Age-dependent Variation in Glutathione Levels

In an attempt to find a possible mechanism for the observed cell-cycle-dependent increase in the OER, we made some preliminary measurements of GSH at various periods during the generation time. Although the constitutive GSH levels varied among experiments, all 3 experiments completed showed a similar qualitative pattern of increase in GSH levels, expressed as GSH equivalents per cell, as the T-1 cells progressed from G_1 phase into late S phase (data not shown). Protein measurements determined in parallel with 1 of these experiments permitted the normalization of GSH levels per milligram cell protein, known to vary in proportion with cell volume (fig. 7). A progressive change in GSH equivalents per milligram cell protein was observed as cells progressed from G_1 to late S phase, with an indication of a decrease late in the cycle. Additional experiments are planned that will permit further quantitative assessment of age-dependent fluctuations in GSH concentration with survival OER in human T-1 cells.

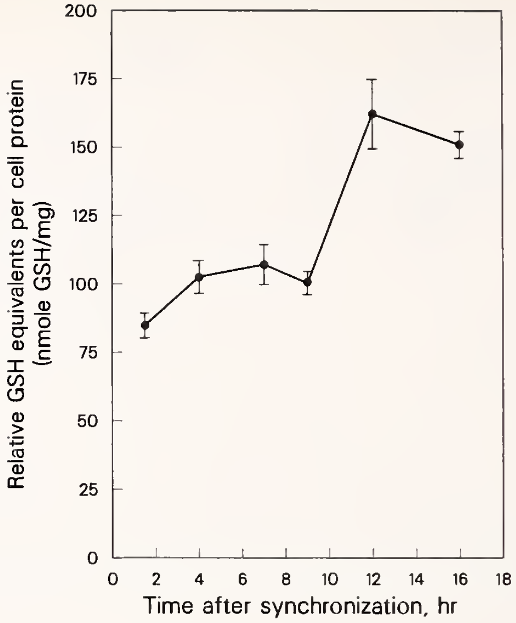


FIGURE 7.—Relative GSH equivalents (nanomoles) per milligram cell protein (±SE) measured in synchronized cell populations at various times during the cell cycle.

DISCUSSION

Survival Oxygen Enhancement Ratio and the Cell Cycle

We have reexamined the question of possible variations in aerobic and hypoxic radiosensitivity during the cell division cycle. We observed an increase in the OER value for x-irradiated human T-1 cell fibroblasts in vitro that was approximately linear as the cells progressed from early G_1 through G_2 phase. The increase in OER was significant based on 95% confidence limits at both the 10% and 1% survival level.

A few reports in the literature (*7–13*) describe experiments designed to measure the OER in synchronously dividing mammalian cells in vitro with x-rays. Researchers find these experiments difficult to complete because there

TABLE 1.—Oxygen enhancement ratios[a]

	Time after synchronization, hr					
Survival level	3	5	8.1	11	14	17
10%	2.58±0.15	2.47±0.27	2.75±0.16	2.73±0.09	2.98±0.12	3.22±0.24
1%	2.57±0.08	2.56±0.13	2.58±0.08	2.76±0.04	2.86±0.06	2.99±0.15

[a] Values include±95% confidence interval.

are many experimental variables that are hard to control. The published reports are divided between those investigators who report that OER values do change through the cell cycle (*7,12,13*) and those who report that they do not (*8–11*). Comparisons are confounded by experimental differences including the use of drugs in some cases to induce synchrony, the manner in which hypoxia was achieved, and the cell line that was studied. For example, Legrys and Hall (*9*) used Chinese hamster cells partially synchronized with hydroxyurea. Hypoxia was achieved by outgassing a Lucite jig holding the cells grown on plastic petri dishes. They (*9*) reported that the pattern of response to a single dose of 8 Gy in air or 20 Gy under hypoxia was very similar, which implied no significant change in the OER with stage in the generation cycle. Complete dose–response curves yielded OER values of 2.6 for populations synchronized in M or S phase (*9*). Other reports in the literature (*8,10,11*) with different biologic and physical systems confirm their observations.

On the other hand, the OER variations reported by us now are in agreement with those of other investigators (*7,12,13*), who found reduced OER values in the early to mid-G_1 phase of the cell cycle. In particular, we confirm the work of Pettersen et al. (*13*) who studied human NHIK 3025 carcinoma cells of the uterine cervix. The NHIK cells were synchronized by mitotic selection without drugs and were degassed and x-irradiated in glass vessels (*13*). The sensitizing effect of oxygen on cells in mid-G_1 phase was found also to increase with increasing dose (*13*). Sapozink (*12*) reported a significantly lower OER early in the cell cycle of HeLa cells synchronized with calcium-free medium and made hypoxic with culturing in a dense feeder cell population. Hence researchers in 3 laboratories who used hamster cells with a short (1.5 hr) G_1-phase duration reported negligible changes in x-ray OER survival as a function of cell age, whereas those in 3 others, using human cells with longer G_1 phases, demonstrated changes through the cell cycle.

Survival Oxygen Enhancement Ratios and Glutathione

Several recent reviews (*22–26*) have been published on the importance of thiols to radiosensitivity and chemosensitivity. Cellular GSH levels have been shown to have a major influence on the radiation response of asynchronous mammalian cells irradiated under hypoxic conditions (*27,28*). Several lines of experimental evidence support this conclusion. Cell lines derived from individuals with genetic defects in GSH metabolism (primarily altered levels of the enzyme GSH synthetase) have provided mutant cell lines with decreased intracellular GSH levels. These cell lines have been reported to exhibit a reduced OER, principally caused by a greater sensitization of the hypoxic compared with the aerobic response (*29–32*). It is acknowledged, however, that it cannot be ruled out that these cell lines (as well as other mutant GSH-deficient cell lines) may also have a deficiency in some other biochemical system responsible for the reduction in their OER values.

In a second type of approach, some have used thiol-modifying agents to evaluate the importance of GSH in the radiation response. Agents like *N*-ethylmaleimide, diethyl maleate, and L-buthionine sulfoximine have been used to decrease intracellular GSH levels with some commensurate increase in the sensitization of the hypoxic radiation response (*33–35*). The effect of thiol-depleting agents on the aerobic response is less clear. In those studies, when no aerobic sensitization occurs, the OER is reduced by the radiosensitizing agents (*33,35*), whereas other investigators report no appreciable alteration in the OER due to simultaneous aerobic and hypoxic sensitization (*36*) or only after conditions of long-term incubation in L-buthionine sulfoximine (*37*) or combined L-buthionine sulfoximine and diethyl maleate treatment (*38*). It might be expected that intracellular concentrations of these agents will be uncertain and that the biochemical reaction may not be fully known.

A third approach was used by Russo et al. (*39*) in an attempt to elevate intracellular thiol levels and measure the effect on the OER. Intracellular thiol levels in Chinese hamster V79 cells were increased 200% upon the addition of 2-oxothiazolidine-4-carboxylate, with a corresponding increase in the OER produced by protection of the hypoxic response with no effect on the aerobic response (*39*). Based on this report, a modification of intracellular thiol levels would predict a commensurate change in the hypoxic x-ray response and in certain instances the OER value.

Variations in Glutathione During the Cell Cycle

In the late 1960s and early 1970s, several reports were published that indicated a diversity of observations including: 1) no consistent change in NPSH content between late G_1- and late S-phase cells (*40–43*), 2) a trend of increasing NPSH levels with cell progression from early G_1-phase through late S-phase cells (*42,44*), or even 3) an increasing trend from G_1 to early S phase and then decreasing values with cell aging from early to late S phase (*45*). It is worth noting that the methods researchers used to measure GSH were either Ellman's technique (*44*) or [^{14}C]*N*-ethylmaleimide binding (*40–43,45*). Harris and Teng (*42*), using the latter binding technique, only found an age-dependent increase in NPSH if they studied G_1-phase cells released from plateau phase. They did not see an increased NPSH in synchronized populations mitotically selected with demecolcine and then allowed to progress.

Our preliminary measurements of GSH made with the Tietze assay on mitotically selected synchronized populations indicated an increase in GSH, normalized on a per total cell protein basis, as the cells progress from early G_1 to late G_2 phase. The increase in intracellular GSH levels appears to correlate with the approximately linear increase in OER in cells aging over the same period.

CONCLUSIONS AND IMPLICATIONS FOR FUNDAMENTAL MECHANISMS

Oxygen can be an effective modifier of radiation-induced damage. Most importantly, oxygen competes with endogenous reducing agents such as GSH in reactions with radiation-induced free radical sites in vital cellular molecules. For sparsely ionizing radiation, a certain number of the DNA radical sites of damage may be restored by reducing species such as GSH, unless oxygen has fixated them first.

In normally aerated Chinese hamster cells, subjected to a 20% oxygen atmosphere, a full OER characteristic to the system can be obtained when stringent hypoxia is administered for the irradiation. If, however, hypoxic cells are supplied with oxygen (1%–50%) in less than 5 mseconds prior to irradiation, only a partial but constant OER of 1.7 is obtained (*46,47*). The explanation has been that there are two components to the oxygen effect. One target, possibly the membrane, to which oxygen diffuses readily would account for the 1.7 portion of the full OER, and another target, which is possibly nuclear DNA, would involve the rest of the full OER value, which is approximately 2.8 to 3.0 in many mammalian cells. If these theoretical targets varied during the cell cycle or if the concentration of a compound such as GSH varied during the cell progression cycle, this might reflect a variable OER during the generation cycle.

In addition to providing a potential modification of both the initial damage yields and spectrum, GSH levels or, more relevantly, GSH contribution to the redox state, may play an important role in the cells' capacity to repair radiation injury. Clearly, further work in this area is needed. It is also important, in our opinion, for one to realize that studies designed to explore the mechanisms of thiol-dependent responses and/or chemoresponses of cells may be confounded by the use of asynchronous populations.

REFERENCES

(*1*) Gray LH: Actions of radiations on living cells. Br J Radiol 25:235–244, 1952.

(*2*) Terasima T, Tolmach LJ: Changes in X-ray sensitivity of HeLa cells during the division cycle. Nature 190:1210–1211, 1961.

(*3*) Terasima T, Tolmach LJ: Variations in several responses of HeLa cells to X-irradiation during the division cycle. Biophys J 3:11–33, 1963.

(*4*) Elkind MM, Whitmore GF: The Radiobiology of Cultured Mammalian Cells. New York: Gordon & Breach, 1967, pp 85–103.

(*5*) Sinclair WK: Cyclic X-ray responses in mammalian cells in vitro. Radiat Res 33:620–643, 1968.

(*6*) Sinclair WK: Cell cycle dependence of the lethal radiation response in mammalian cells. Curr Top Radiat Res Q 7:264–285, 1972.

(*7*) Berry RJ: A reduced oxygen enhancement ratio for X-ray survival of HeLa cells in vitro, after treatment with methotrexate. Nature 208:1108–1109, 1965.

(*8*) Kruuv J, Sinclair WK: X-ray sensitivity of synchronized Chinese hamster cells irradiated during hypoxia. Radiat Res 36:45–54, 1968.

(*9*) Legrys GA, Hall EJ: The oxygen effect and X-ray sensitivity in synchronously dividing cultures of Chinese hamster cells. Radiat Res 37:161–172, 1969.

(*10*) Kimler BF, Sinclair WK, Elkind MM: *N*-Ethylmaleimide sensitization of X-irradiated hypoxic Chinese hamster cells. Radiat Res 71:204–213, 1977.

(*11*) Chapman JD, Webb RG, Borsa J: Radiosensitization of mammalian cells by *p*-nitroacetophenone. I. Characterization in asynchronous and synchronous populations. Int J Radiat Biol 19:561–573, 1971.

(*12*) Sapozink MS: Oxygen enhancement ratios in synchronous HeLa cells exposed to low-LET radiation. Radiat Res 69:27–39, 1977.

(*13*) Pettersen EO, Christensen T, Bakke O, et al: A change in the oxygen effect throughout the cell cycle of human cells of the line NHIK-3025 cultivated in vitro. Int J Radiat Biol 31:171–184, 1977.

(*14*) Palcic B, Skarsgard LD: Reduced oxygen enhancement ratio at low doses of ionizing radiation. Radiat Res 100:328–339, 1984.

(*15*) Palcic B, Brosing JW, Skarsgard LD: Survival measurements at low doses: Oxygen enhancement ratios. Br J Cancer 46:980–984, 1984.

(*16*) Blakely EA, Tobias CA, Yang TCH, et al: Inactivation of human kidney cells by high energy monoenergetic heavy-ion beams. Radiat Res 80:122–160, 1979.

(*17*) Blakely EA, Chang PY, Lommel L: Cell-cycle dependent recovery from heavy-ion damage in G_1-phase cells. Radiat Res 104:s-145–s-157, 1985.

(*18*) Tietze F: Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. Anal Biochem 27:502–522, 1969.

(*19*) Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254, 1976.

(*20*) Cleland WW: The statistical analysis of enzyme kinetic data. Adv Enzymol 29:1–32, 1967.

(*21*) Albright NW: Computer programs for the analysis of cell survival date. Radiat Res 112:331–340, 1987.

(*22*) Vos O, van der Schans GP, Roos-Verhey WSD: Reduction of intracellular glutathione content and radiosensitivity. Int J Radiat Biol 50:155–165, 1985.

(*23*) Edgren MR, Solen G, Revesz L: Specificity of endogenous glutathione in determining the oxygen enhancement of cellular radiosensitivity. Int J Radiat Oncol Biol Phys 12:1147–1150, 1986.

(*24*) Clark EP: Thiol induced biochemical modification of chemo- and radioresponses. Int J Radiat Oncol Biol Phys 12:1121–1126, 1986.

(*25*) Biaglow JE, Varnes ME, Clark EP, et al: The role of thiols in cellular response to radiation and drugs. Radiat Res 95:437–455, 1983.

(*26*) Durand RE: Roles of thiols in cellular radiosensitivity. Int J Radiat Oncol Biol Phys 10:1235–1236, 1984.

(*27*) Cullen BM, Michalowski A, Walker H, et al: Studies on the radiobiological oxygen constant, K, for mammalian cells: Correlation between K and the non-protein sulfhydryl content of mammalian cells. Int J Radiat Biol 38:525–536, 1980.

(*28*) Hodgkiss RJ, Jones NR, Watts ME, et al: Glutathione depletion enhances the lifetime of oxygen-reactive radicals in mammalian cells. Int J Radiat Biol 46:673–674, 1984.

(*29*) Malaise EP: Reduced oxygen enhancement of the radiosensitivity of glutathione-deficient fibroblasts. Radiat Res 95:486–494, 1983.

(*30*) Revesz L: The role of endogenous thiols in intrinsic radioprotection. Int J Radiat Biol 47:361–368, 1986.

(*31*) Debieu D, Deschavanne PJ, Midander J, et al: Survival curves of glutathione synthetase deficient human fibroblasts: Correlation between radiosensitization in hypoxia and glutathione synthetase activity. Int J Radiat Biol 48:525–543, 1985.

(*32*) Astor MB: Oxygen concentration and the OER for acutely or chronically thiol deficient cells. Int J Radiat Oncol Biol Phys 12:1131–1134, 1986.

(*33*) Bump EA, Yu NY, Brown JM: Radiosensitization of hypoxic tumor cells by depletion of intracellular glutathione. Science 217:544–545, 1982.

(*34*) Koch CJ, Stobbe CC, Baer KA: Combined radiation-protective and radiation-sensitizing agents. III. Radiosensitization by misonidazole as a function of concentration of endogenous glutathione or exogenous thiols. Int J Radiat Oncol Biol Phys 12:1151–1155, 1986.

(*35*) Clark EP, Epp ER, Biaglow JE, et al: Glutathione depletion, radiosensitization, and other misonidazole potentiation in hypoxic Chinese hamster ovary cells by buthionine sulfoximine. Radiat Res 98:370–380, 1984.

(*36*) Mitchell JB, Russo A, Biaglow JE, et al: Cellular glutathione depletion by diethyl maleate or buthionine sulfoximine: No effect of glutathione depletion on the oxygen enhancement ratio. Radiat Res 96:422–428, 1983.

(*37*) Astor MB, Hall EJ, Biaglow JE, et al: Effects of D,L-buthionine-*S,R*-sulfoximine on cellular thiol levels and the oxygen effect in Chinese hamster V79 cells. Int J Radiat Oncol Biol Phys 10:1239–1242, 1984.

(*38*) Biaglow JE, Clark EP, Epp ER, et al: Nonprotein thiols and the radiation response of A549 human lung carcinoma cells. Int J Radiat Biol 44:489–495, 1983.

(*39*) Russo A, Mitchell JB, Finkelstein E, et al: The effects of cellular glutathione elevation on the oxygen enhancement ratio. Radiat Res 103:232–239, 1985.

(*40*) Klein P, Robbins E: An ultrasensitive assay for soluble sulfhydryl and its application to the study of glutathione levels during the HeLa life cycle. J Cell Biol 46:165–168, 1970.

(*41*) Harris JW, Painter RB, Hahn GM: Endogenous non-protein sulfhydryl and cellular radiosensitivity. Int J Radiat Biol 15:289–292, 1969.

(*42*) Harris JW, Teng SS: Sulfhydryl groups during the S-phase: Comparison of cells from G_1, plateau-phase G_1, G_0. J Cell Physiol 81:91–96, 1972.

(*43*) Harris JW: Non-protein sulfhydryl content of Ehrlich ascites tumor cells exposed to hyperbaric oxygen or X-irradiation. Exp Cell Res 50:293–305, 1968.

(*44*) Ohara H, Terasima T: Variations of cellular sulfhydryl content during cell cycle of HeLa cells and its correlation to cyclic change of X-ray sensitivity. Exp Cell Res 58:182–185, 1969.

(*45*) Sinclair WK: Modification of cell cycle response of synchronous mammalian cells to ionizing radiation by inhibition of repair. *In* Control of Proliferation in Animal Cells. Cold Spring Harbor Conferences on Cell Proliferation, vol 1. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1974, pp 985–994.

(*46*) Shenoy MA, Asquith JC, Adams GE, et al: Time-resolved oxygen effects in irradiated bacteria and mammalian cells: A rapid-mix study. Radiat Res 62:498–512, 1975.

(*47*) Michael BD, Harrop HA: Time scale and mechanism of radiosensitization and radioprotection at the cellular level. *In* Proceedings of the Key Biscayne Conference on Combined Modalities in Cancer Treatment: Radiation Sensitizers and Protectors (Brady LW, ed). New York: Masson, 1980, pp 14–22.

Inhibition of Radiation-induced DNA–Protein Cross-link Repair by Glutathione Depletion With L-Buthionine Sulfoximine[1]

Nancy L. Oleinick,[2,*] Liang-yan Xue,[2] Libby R. Friedman,[2] Laurie L. Donahue,[2] and John E. Biaglow[2,3]

ABSTRACT—Cells depleted of their glutathione (GSH) by treatment with L-buthionine sulfoximine (BSO) are more sensitive to ionizing radiation and chemotherapeutic agents. To assess the effects of GSH depletion on repair of radiation-induced DNA damage, we have determined DNA–protein cross-links (DPC) in A549 cells by a nitrocellulose filter binding assay. Untreated A549 cells have a low level of DPC (0.7%), whereas cells incubated with BSO for 92 hours have an elevated level of DPC (1.5%). The dose response for production of radiation-induced DPC is approximately 0.4% DNA bound per 10 Gy for both BSO-treated and non-pretreated cells. Cells not exposed to BSO repair 85% of the radiation-induced DPC in 4 hours. Less repair (55%) is observed for BSO-treated cells incubated for 4 hours postirradiation in conditioned medium, and DPC repair is nearly completely blocked if GSH-depleted cells are given fresh medium during the 4-hour repair interval. The DPC repair is not influenced by the pH of the media between 6.6 and 7.4. Data indicate that intracellular GSH regulates the level of a sulfhydryl-containing repair enzyme, and that other, as yet undefined, nutrients may further influence the steady-state level of DPC.—NCI Monogr 6:225–229, 1988.

Recently, there has been considerable interest in the role of cellular nonprotein sulfhydryls, especially GSH, in radiation response. It has been shown that depletion of intracellular GSH by diethyl maleate or BSO increases the sensitivity of hypoxic cells to radiation (*1–4*). Aerobic sensitization has also been observed upon depletion of GSH from V79 (*5*) or A549 (*1,6*) cells. In addition to diethyl maleate and BSO, other agents, such as diamide (*7,8*), *N*-ethylmaleimide (*9*), and dimethyl fumarate (*10*) have been used to deplete intracellular GSH. Most of the above investigators examined the effects of the various agents on cell survival. The induction and repair of single-strand breaks were measured by Edgren et al. (*11*) in normal human fibroblasts and in fibroblasts from patients with a genetic deficiency in GSH synthesis. The GSH deficiency was associated with a greater than normal yield of single-strand breaks in anoxia and a slower repair rate for irradiation in air.

When cells are exposed to gamma or UV radiation or are treated with some chemicals, DPC are produced (*12–15*). According to our earlier results (*16*), the yield of cross-linked DNA is approximately linear with dose within the dose range of 0–100 Gy of gamma radiation. The DPC are formed preferentially between regions of DNA containing actively transcribing sequences and proteins of the nuclear matrix (*17*). Those DNA regions encompassing active genes are also more susceptible to single-strand break formation than is the bulk DNA (*18*). Thus nuclear matrix-associated DNA may be a radiosensitive fraction of DNA in the eukaryotic nucleus, and DPC may be an important biologic indicator of radiation damage. We have used a filter-binding assay to quantify DPC. This simple and reliable method depends upon the strong affinity of nitrocellulose filters for proteins, but not for free DNA. In the present study, we have determined the effects of GSH depletion on the formation and repair of DPC by gamma radiation.

MATERIALS AND METHODS

Cell culture.—The A549 human lung carcinoma cells were grown as monolayers in 9-cm petri dishes in McCoy's 5A medium supplemented with 5% fetal bovine serum and 10% calf serum and buffered with 0.02 *M* HEPES, pH 7.2 (*1*) at 37° C in an atmosphere of 95% air and 5% CO_2. Some cultures received 0.1 m*M* BSO 4 hours after plating. Forty-two hours prior to use, the cells were labeled with [methyl-^{3}H]thymidine (0.1 μCi/ml). All cultures were in an active exponential phase of growth at the time of experimentation.

Irradiation and incubation.—For irradiation, the medium was removed and replaced with 5 ml cold HEPES-buffered Hanks' balanced salt solution. Cultures were irradiated on ice in air with a cobalt-60 irradiator at a dose rate of 11 Gy/minute. Immediately after irradiation, the balanced salt solution was replaced with either F-medium or the culture's C-medium, and the cultures were returned to the 37° C incubator for various lengths of time. Additional modifications of the incubation medium are described in the figure legends.

Assay for DNA–protein cross-links.—The DPC were determined by a nitrocellulose filter-binding technique (*16*). Briefly, cells were lysed in standard saline citrate (0.15 *M* NaCl, 15 m*M* sodium citrate) containing 1% sarkosyl, 1 *M* sodium perchlorate, and 5 m*M* EDTA. After heating at

---

ABBREVIATIONS: GSH = glutathione; BSO = L-buthionine sulfoximine; DPC = DNA–protein cross-links; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; F-medium = fresh McCoy's 5A medium with serum; C-medium = conditioned medium.

[1] Supported by Public Health Service grants CA-15378 and CA-13747 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Division of Biochemical Oncology, Department of Radiology, Case Western Reserve University School of Medicine, Cleveland, OH.

[3] *Present address:* Department of Radiation Therapy, University of Pennsylvania, Philadelphia, PA 19104.

* *Reprint requests:* Nancy L. Oleinick, Ph.D., Division of Biochemical Oncology, Wearn Research Laboratory, Case Western Reserve University School of Medicine, 2058 Abington Rd., Cleveland, OH 44106-2699.

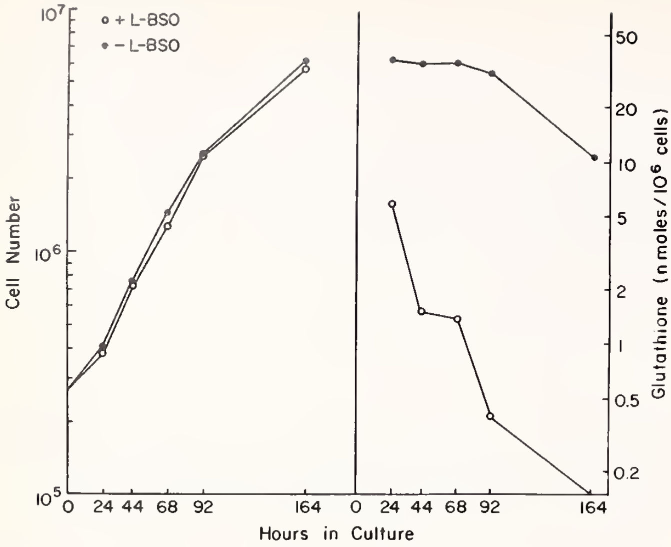


FIGURE 1.—Growth and GSH level of cells cultured with or without 0.1 m*M* BSO. The A549 cells were plated in 9-cm petri dishes ($\sim 2.7 \times 10^5$ cells/dish). Some cultures were given 0.1 m*M* BSO 4 hr later (○), and others received no BSO (●). At various times, cultures were trypsinized and cell number/dish was determined; other cultures were processed for measurement of GSH.

60° C for 20 minutes and cooling, the lysate was filtered through a nitrocellulose filter. Radioactivity on the washed filters was determined in a liquid scintillation counter.

Determination of glutathione.—We used the Tietze assay (*19*) to determine the amount of GSH in the cells after 1–7 days of growth in the presence or absence of BSO.

RESULTS AND DISCUSSION

Figure 1 shows the cell numbers and GSH levels of cultures grown in the presence or absence of 0.1 m*M* BSO. The cell counts were almost identical for control and BSO-treated cultures, which indicated no effect of GSH depletion on cell growth during the period studied. However, the GSH level of BSO-treated cells became rapidly depleted with time, whereas the control cells showed little or no change in GSH level. After 24, 44, 68, and 92 hours of BSO treatment, the GSH level was 16.3%, 4.4%, 4.0%, and 1.3%, respectively, of the corresponding control values. When grown in medium with BSO for 164 hours, the GSH dropped to less than the smallest detectable amount.

The radiation dose response for production of DPC was determined in untreated and BSO-treated cultures (fig. 2). For control and BSO-treated cells, the yield of cross-linked DNA increases linearly with radiation dose. The calculated slopes for the two dose–response curves are 0.37% and 0.43% DNA bound/10 Gy, respectively. Thus the response to irradiation for formation of DPC is not significantly different in the two culture conditions. However, a consistent difference was observed in the background levels of DPC from unirradiated cells: The percent DNA retained on the filters is 1.47% after 92 hours in BSO compared with 0.70% without BSO. Thus depletion of GSH with BSO can elevate the normal background level of DPC. The ability of thiol-reactive agents to elevate DPC was initially reported by Grunicke et al. (*20*). It is not yet known whether the normal, BSO-induced, and radiation-induced DPC are identical structurally or mechanistically.

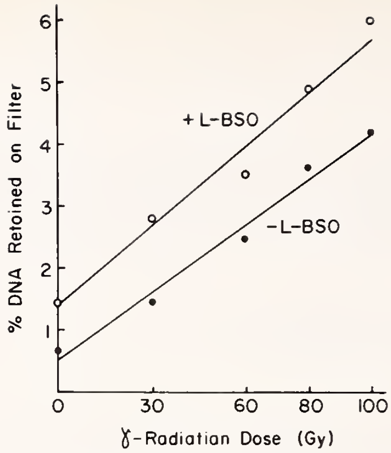


FIGURE 2.—Dose response for induction of DPC in untreated and BSO-treated A549 cells. Cultures were grown with (○) or without (●) 0.1 m*M* BSO for 92 hr and were labeled with [^{3}H]thymidine 42 hr prior to irradiation. Cells were harvested immediately thereafter and assayed for DPC. *Points* represent the average of duplicate determinations, and the line was calculated by the least-squares method.

The kinetics of the release of DPC was followed during a 4-hour postirradiation incubation at 37° C in control and BSO-pretreated cells (fig. 3). Because the efficiency of repair of DPC might depend upon the level of GSH at the time of irradiation, the kinetics of repair were determined after 1, 3, 4, and 7 days treatment with 0.1 m*M* BSO. In these experiments, the cells were irradiated in Hanks' balanced salt solution on ice, after which they were incubated in C-medium. With the exception of cells treated with

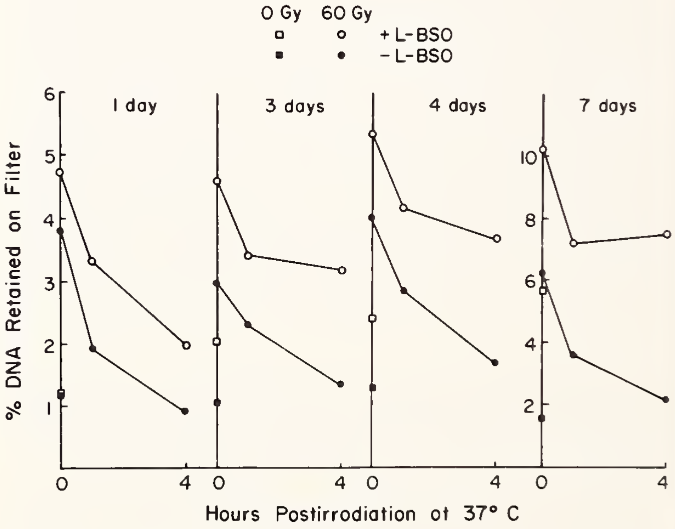


FIGURE 3.—Postirradiation removal of DPC. Cultures incubated in medium with (○) or without (●) 0.1 m*M* BSO, for 1 day, or 3, 4, or 7 days were exposed to 60 Gy gamma radiation in complete Hanks' balanced salt solution or were not irradiated (□, ■). Cells were either harvested immediately after irradiation or were incubated in their C-medium (±BSO) at 37° C for the times indicated, before harvest and assay.

TABLE 1.—Percent repair of DPC in control and BSO-treated cells[a]

Cells	Days in culture	DNA retained on filter, % 0 Gy (A)	60 Gy, 0 hr (B)	60 Gy, 4 hr (C)	Repair, % (B − C)/(B − A)
Control	1	1.18	3.81	0.90	100
	3	1.06	2.96	1.35	83.8
	4	1.31	3.99	1.69	85.3
	7	1.54	6.23	2.20	85.9
BSO-treated	1	1.17	4.76	1.97	77.7
	3	2.05	4.59	3.17	55.6
	4	2.40	5.35	3.68	55.3
	7	5.74	10.20	7.49	60.8

[a] Cells were exposed to 60 Gy and incubated for 4 hr at 37° C. The data of fig. 3 have been used to calculate the percent of 60 Gy-induced DPC repaired in 4 hr in BSO-treated and untreated A549 cells.

BSO for only 1 day, the repair of radiation-induced DPC appeared to be less extensive in BSO-pretreated cells compared with control cells without BSO. The initial phase of the repair during the first hour seemed to progress at the same rate irrespective of the presence of BSO. No significant change in the background level of DPC was observed during the 4-hour postirradiation period (data not shown).

To evaluate more fully the differences between BSO-pretreated and control cultures, we calculated the percentage of radiation-induced DPC that had been repaired by 4 hours in each case (table 1). The percent repair was calculated as (B − C)/(B − A), where A is the percent DNA in DPC in unirradiated cells, B is the initial level of DPC after 60 Gy, and C is the residual level of DPC after the cells are incubated in C-medium for 4 hours. The percent removal of cross-linked DNA was consistently lower in BSO-treated than in non-BSO-treated cells. Whereas the control cells were able to remove 80%–100% of the radiation-induced DPC in 4 hours, cells treated for 3 or more days with BSO removed only 55%–60% of the DPC induced by 60 Gy. The depressed GSH levels appear to be associated with a reduced capacity to remove cross-linked DNA.

To control for the possibility that BSO was directly inhibiting repair of DPC, we irradiated control cells, grown without BSO, with 60 Gy and then incubated them for 4 hours in C-medium to which 0.1 m*M* BSO had been added. The GSH level of that culture was reduced only 7% during the 4-hour incubation period, and the culture was as efficient in removing DPC as companion cultures grown and incubated postirradiation without BSO (data not shown). Therefore, BSO per se has no direct effect on repair of DPC. Rather, intracellular GSH appears to play an important role in the repair of DPC.

We previously reported the results of another series of experiments showing a nearly complete absence of repair of DPC in BSO-treated A549 cells (*21*). In those experiments, the cells had been incubated postirradiation in F-medium. Thus we next compared the effects of F- versus C-medium postirradiation. When non-BSO-treated cells were incubated after irradiation in F-medium instead of C-medium, little difference was observed in the rate or extent of repair of radiation-induced DPC (fig. 4). In contrast, for BSO-pretreated cells, the repair of DPC in F-medium, with or without 0.1 m*M* BSO, was observed to be much slower than repair in C-medium containing 0.1 m*M* BSO. No changes in background levels of DPC were observed in unirradiated cells subjected to similar medium transfers (data not shown).

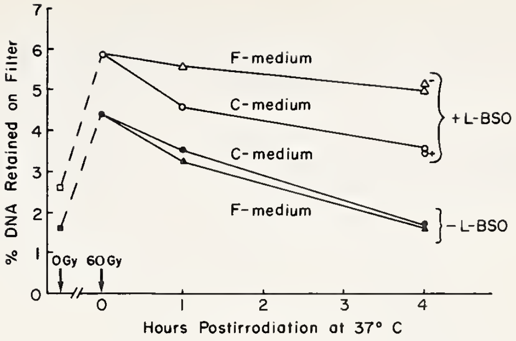


FIGURE 4.—Comparison of the effects of F- and C-medium on the kinetics of the release of DPC following 60 Gy gamma radiation. The A549 cells were grown for 4 days with or without 0.1 m*M* BSO, and then some cultures were given 60 Gy. After irradiation, the cells were incubated at 37° C for up to 4 hr in F- (△, ▲) or C-medium (○, ●) as indicated. Some cultures which had been pretreated with BSO received F-medium containing fresh 0.1 m*M* BSO for postirradiation incubation (△), whereas others were incubated in F-medium without BSO (△⁻). In addition, some BSO-pretreated cultures were incubated postirradiation in their C-medium (+BSO) to which 0.1 m*M* fresh BSO was added (○+).

One obvious difference between the two media is the pH. The F-medium was routinely adjusted to pH 7.2, whereas the pH of C-medium dropped to 6.8 during the 3- to 4-day growth period. Therefore, we examined the effects of pH of the medium on the repair of cross-linked DNA. The results shown in figure 5 indicate that the difference in extent of repair of DPC in BSO-treated cells in F- or C-medium is not related to the pH of medium. Media were adjusted to pH 6.6, 6.9, 7.2, or 7.4 before being added to irradiated cells. After 4 hours, media added to the cells at pH 6.6 had risen to pH 6.8–6.9, whereas the pH of the most alkaline

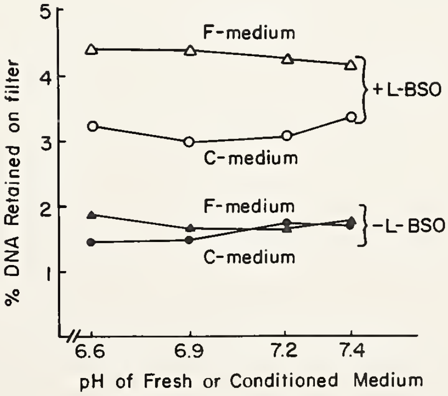


FIGURE 5.—Effects of pH on repair of DPC. Cultures grown in medium with or without 0.1 m*M* BSO for 4 days were exposed to 60 Gy gamma radiation. After irradiation, cells were incubated at 37° C for 4 hr in either F- (△, ▲) or C-medium (○, ●) adjusted to the indicated pH.

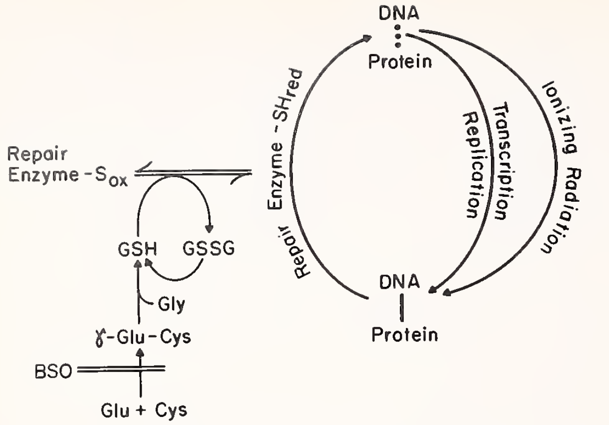


FIGURE 6.—Formation and removal of DPC in A549 cells and the effect of BSO. S_{ox} = oxidized (disulfide) form of sulfhydryl in enzyme; GSSG = oxidized glutathione; Cys = cysteine.

media had dropped to pH 7.2. However, the repair of DPC in BSO-treated cells was always slower in F- than in C-medium.

We also tested the possibility that the addition of fresh BSO with the F-medium was inhibitory to DPC repair. Cells pretreated with BSO were incubated postirradiation in F-medium either with or without 0.1 m*M* BSO or in C-medium (containing the BSO present during the 4 days of cell culture) with or without the further addition of 0.1 m*M* fresh BSO (fig. 4) for 4 hours after irradiation. There was always a difference in repair of BSO-pretreated cells between the media. However, the addition of fresh BSO to either medium had little or no effect on the repair efficiency. Thus the reduced capacity of BSO-pretreated cells to repair DPC in F-medium must be due to the inhibition of a step in repair by some component(s) of fresh McCoy's 5A, or serum, or the acceleration of repair by a component(s) of the C-medium. The component(s) appear(s) to be effective only in cells with a suboptimal GSH level.

The diagram in figure 6 presents a model to explain the results reported in this study. A steady-state background level of DPC is the result of tight binding of transcribing and replicating DNA regions to a subset of proteins of the nuclear matrix (*17*), and turnover of these complexes catalyzed by a sulfhydryl-dependent (repair) enzyme(s). The background level is elevated in GSH-depleted cells because of a reduced ability to maintain total protein thiols in general (*21*) and, in particular, to maintain the putative repair enzyme in its reduced (sulfhydryl) active form. Additional DNA regions enriched in active genes become cross-linked to matrix proteins by ionizing radiation, and their repair is likewise compromised when the intracellular GSH level is depressed. The further reduction in apparent repair of radiation-induced DPC observed when BSO-treated cells are incubated in F-, as opposed to C-medium, could be due to the stimulation of cell growth by component(s) of F-medium, leading to more replication- and transcription-associated DPC that compete with radiation-induced DPC for the minimally active repair system. Although mechanistic details are not clear at present, the inhibition of repair of DPC may contribute to the increased radiation sensitivity of GSH-depleted cells.

REFERENCES

(*1*) BIAGLOW JE, CLARK EP, EPP ER, et al: Nonprotein thiols and the radiation response of A549 human lung carcinoma cells. Int J Radiat Biol 44:489–495, 1983.

(*2*) BUMP EA, YU NY, BROWN JM: Radiosensitization of hypoxic tumor cells by depletion of intracellular glutathione. Science 217:544–545, 1982.

(*3*) BUMP EA, YU NY, BROWN JM: The use of drugs which deplete intracellular glutathione in hypoxic cell radiosensitization. Int J Radiat Oncol Biol Phys 8:439–442, 1982.

(*4*) MITCHELL JB, RUSSO A, BIAGLOW JE, et al: Cellular glutathione depletion by diethyl maleate or buthionine sulfoximine: No effect of glutathione depletion on the oxygen enhancement ratio. Radiat Res 96:422–428, 1983.

(*5*) ASTOR MB, HALL EJ, BIAGLOW JE, et al: Effects of D,L-buthionine-*S,R*-sulfoximine on cellular thiol levels and the oxygen effect in Chinese hamster V79 cells. Int J Radiat Oncol Biol Phys 10:1239–1242, 1984.

(*6*) BIAGLOW JE, VARNES ME, EPP ER, et al: Factors involved in depletion of glutathione from A549 human lung carcinoma cells; implications for radiotherapy. Int J Radiat Oncol Biol Phys 10:1221–1227, 1984.

(*7*) HARRIS JW: Cellular thiols in radiation and drug response; use of specific reagents. *In* Radioprotectors and Anticarcinogens (Nygaard OF, Simic MG, eds). New York: Academic Press, 1983, pp 255–274.

(*8*) SKOV KA, PALCIC B, SKARSGARD LD: Modification of the action of misonidazole. III. The effects of diamide on toxicity and radiosensitization. Int J Radiat Biol 40: 335–345, 1981.

(*9*) KIMLER BF, SINCLAIR WK, ELKIND MM: *N*-Ethylmaleimide sensitization of X-irradiated hypoxic Chinese hamster cells. Radiat Res 71:204–213, 1977.

(*10*) BIAGLOW JE, VARNES ME, ASTOR M, et al: Non-protein thiols and cellular response to drugs and radiation. Int J Radiat Oncol Biol Phys 8:719–723, 1982.

(*11*) EDGREN M, REVESZ L, LARSSON A: Induction and repair of single-strand DNA breaks after X-irradiation of human fibroblasts deficient in glutathione. Int J Radiat Biol 40:355–363, 1981.

(*12*) FORNACE AJ JR, LITTLE JB: DNA crosslinking induced by X-rays and chemical agents. Biochim Biophys Acta 477:343–355, 1977.

(*13*) SMITH KC: Radiation-induced cross-linking of DNA and protein in bacteria. *In* Carcinogenesis and Radiation Biology (Smith KC, ed). New York: Plenum Press, 1976, pp 67–91.

(*14*) FORNACE AJ JR, LITTLE JB: DNA–protein cross-linking by chemical carcinogens in mammalian cells. Cancer Res 39:704–710, 1979.

(*15*) BANJAR ZM, HNILICA S, BRIGGS RC, et al: Crosslinking of chromosomal proteins to DNA in HeLa cells by UV, gamma radiation and some antitumor drugs. Biochem Biophys Res Commun 114:767–773, 1983.

(*16*) CHIU SM, SOKANY NM, FRIEDMAN LR, et al: Differential processing of ultraviolet or ionizing radiation-induced DNA–protein cross-links in Chinese hamster cells. Int J Radiat Biol 46:681–690, 1984.

(*17*) CHIU SM, FRIEDMAN LR, SOKANY NM, et al: Nuclear matrix proteins are crosslinked to transcriptionally active gene sequences by ionizing radiation. Radiat Res 107: 24–38, 1986.

(*18*) CHIU SM, OLEINICK NL, FRIEDMAN LR, et al: Hypersensitivity of DNA in transcriptionally active chromatin to ionizing radiation. Biochim Biophys Acta 699:15–21, 1982.

(*19*) TIETZE F: Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione.

Applications to mammalian blood and other tissues. Anal Biochem 27:502–522, 1969.

(*20*) Grunicke H, Bock KW, Becher H, et al: Effect of alkylating antitumor agents on the binding of DNA to protein. Cancer Res 33:1048–1053, 1973.

(*21*) Biaglow JE, Varnes ME, Tuttle SW, et al: The effect of L-buthionine sulfoximine on the aerobic radiation response of A549 human lung carcinoma cells. Int J Radiat Oncol Biol Phys 12:1139–1142, 1986.

Radioprotection of Rat Subependymal Plate With 4-OH Sodium Butyrate[1]

P. Davey,[2,*] F. Grieve,[1,2] and V. A. Ranaldi[2,3]

ABSTRACT—The central nervous system is a radiation-dose-limiting structure, and cellularity of the rat subependymal plate (a location of neuroglial stem cells) has been used as a model of radiation damage. In the present work, an attempt has been made to improve its radiation tolerance using 4-OH sodium butyrate (γOH). Adult rats received 10-Gy 250-kV (peak) x-rays or 3.5-Gy 15-MeV deuterons plus Be neutrons. Cell counts were obtained by histological examination of the subependymal plate. Photon and neutron irradiation alone resulted in a mean cell depletion of 62% and 58%, respectively, compared with sham-irradiated controls, which was not statistically significant; the relative biologic effectiveness was 2.9. In the absence of radiation, γOH did not significantly alter the cellularity of the subependymal plate, compared with that in controls treated with chloral hydrate. At doses $\geq$ 1 g/kg, γOH was associated with a statistically significant reduction of subependymal plate cell depletion in animals treated with photon or neutron radiation, and the magnitude of the effect was similar. Arterial blood gas analysis failed to show a significant difference in arterial oxygen tension between control and test animals.—NCI Monogr 6:231–234, 1988.

To date, the results of treating astrocytomas with ionizing radiation remain disappointing (*1*). This reflects dose limitations set by normal tissue radiation tolerance in addition to inherent tumor radioresistance (*2*) and tumor hypoxia (*3*). However, attempts to increase tumor radiation dose do appear to result in improved median survival (*4*). A naturally occurring cerebral metabolite, γOH, is used clinically in the management of patients with head injury (*5*) or narcolepsy (*6*). It has been reported to protect mice against TBI with a DRF of ~1.3 (*7*). The rat subependymal plate is considered to be a site of neuroglial stem cells in neonatal and adult animals. A homologous structure has been described in a variety of mammals including adult primates (*8*). There are considerable morphological and kinetic data (*9*), although an in vivo model of cell renewal (*10*) failed to distinguish separate neuroglial lineages (astrocytes and oligodendrocytes). Nevertheless, the rat subependymal plate has been used to assay the effects of photons (*11*), neutrons (*12*), and combinations of photons and methotrexate (*13*). Although it is generally considered that human cerebral radionecrosis primarily reflects vascular damage (*14*), it seems unlikely that this occurs without previous neuroglial stem cell depletion, which may have been expressed as an "early delayed" neurological syndrome (*15*). This study attempts to evaluate γOH as a cerebral radioprotector by using the rat subependymal plate assay.

MATERIALS AND METHODS

Animals.—A breeding colony of Sprague-Dawley rats was established from animals supplied by Bantin and Kingman (Hull, England). Rats were fed CRM(X) pellets (Labsure, Poole, England) and water ad libitum. All experiments involving radiation were carried out on female rats at 20 weeks of age, since subependymal plate cellularity is known to vary with animal strain, age, and sex (*13*).

Radiation.—Photons were produced from a 250-kV (peak) x-ray set. A phantom rat was constructed from pelt, skull, and tissue-equivalent wax, and the photon dose was measured with LiF thermoluminescent dosimetry. Animals were irradiated individually using a specially constructed jig incorporating lead shielding to limit exposure to the cerebrum. Parallel opposed lateral fields were used, initial setups being corroborated by means of check films. The dose rate was 0.61 Gy/minute. Neutrons were generated in a cyclotron (CS30, Cyclotron Corp., Berkeley, CA) by directing a deuteron beam with an energy of 15 MeV against a thick Be target. Dose was measured on a phantom rat with a tissue-equivalent ionization chamber. Parallel opposed lateral fields were used, and the beam was collimated to limit exposure to the cerebrum. The dose rate was 0.25–0.35 Gy/minute. The photon contribution to the total dose was 3%.

Histological evaluation.—Animals were anesthetized with ether, and at the onset of apnea, the thorax was opened via a subdiaphragmatic approach. A 17F plastic cannula (Venflon, Viggo, Swindon, England) was introduced into the left ventricle after the apex of the ventricle was excised; it was advanced into the ascending aorta. Heparinized saline (NaCl, 0.9% weight/vol) was infused and, assuming adequate flow, the catheter was secured with a tie. The right

---

ABBREVIATIONS: γOH = 4-OH sodium butyrate; TBI = total body irradiation: DRF = dose reduction factor(s); RBE = relative biologic effectiveness; PaO_2 = arterial oxygen tension; TCA = tricarboxylic acid.

---

[1] Supported by the Kodak Trust and by the Western General Hospital Cancer Research Fund (*F. Grieve*).

[2] Department of Clinical Oncology, University of Edinburgh, Scotland.

[3] We thank the staff of the Western General Hospital, Edinburgh, for their assistance; Dr. Jerry Williams for performing the dosimetry; Professor J. D. Miller for making a blood gas analyzer available; Dr. John Hopewell (Normal Tissue Radiobiology Research Group, Cancer Research Campaign, Churchill Hospital, Oxford, England) for his advice on setting up the subependymal plate assay; Drs. A. M. Rauth and R. P. Hill (Ontario Cancer Institute, Toronto, Canada) for recommendations regarding data presentation; and Chris-Anne Mckenzie and Rosetta Walker for performing the histologic processing, which was supported by the Western General Hospital Cancer Research Fund.

* *Reprint requests:* P. Davey, M.B., The Ontario Cancer Institute, 500 Sherbourne St., Toronto, Ontario M4X 1K9, Canada.

atrium was incised to allow free drainage of venous blood. Irrigation was continued until blood no longer drained. Fixative solution (formaldehyde, 5.5% vol/vol, with ammonium bromide, 2% weight/vol) was then infused; 400 ml was delivered over 20–30 minutes. Brains were then removed intact. Following resection of the cerebellum, the cerebrum was bivalved in a horizontal plane as previously described (*16*) and was stored in dilute formaldehyde solution (3.8% vol/vol) before processing. An automatic tissue processor was used. Tissue was treated sequentially with 70% alcohol for 1 hour; 80% alcohol for 2 hours; 90% alcohol for 2 hours; 74 over proof industrial methylated spirit for 2 hours; absolute alcohol for 2 hours (three changes); and chloroform for 6 hours (three changes). Embedding was in paraffin wax, and 4-μm histological sections were cut and stained with hematoxylin and eosin.

Total subependymal cell nuclei counts were obtained at magnification × 400, with a 0.1-mm net graticule incorporated in the microscope eyepiece. The graticule was aligned with the tip of the lateral ventricle, and all nuclei within the subependymal plate rostral to that point were included. Normally, only intact nuclei were scored; however, pyknotic nuclei evident in the sections of brains irradiated the same day the rats were killed were included. Four sections (two per hemisphere) from each brain were assessed. Histological sections that did not conform with the reference plane were discarded. A mean cell count was obtained for each animal. An examination showed that cell counts of pooled sham-irradiated animals approached a normal distribution (data not shown). Means of animal groups were compared using Student's *t*-test. Data have been presented as the means of the groups ± SE of the means.

Blood gas analysis.—Samples of arterial blood were obtained from the left ventricle as described by Waynforth (*17*) and assayed using an automatic blood gas analyzer (model 1302; Instrument Laboratory Systems, Warrington, England).

RESULTS

Initial experimentation was undertaken to determine cellular depletion of the subependymal plate as a function of time. Chloral hydrate anesthesia (Sigma Chemical Co., St. Louis, MO; 0.3 g/kg, 10% weight/vol) was given ip, and groups of 5 animals were sham irradiated and killed on the day of sham irradiation (after ~3 hr) and at weekly intervals thereafter for 10 weeks. Similar groups of 5 animals were each exposed to 10-Gy photons or 3.5-Gy neutrons. One group received 6-Gy photons. The doses of photons and neutrons selected were based on the assumptions that a dose of 10-Gy photons 1) approximates the human tolerance for whole brain single-dose radiation; 2) is the limiting dose above which the subependymal plate (in the rat) does not recover; and 3) would be biologically isoeffective with 3.5-Gy neutrons.

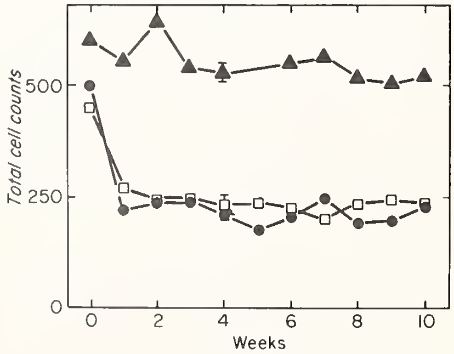


FIGURE 1.—Depletion of rat subependymal plate as function of time following sham irradiation (▲) or irradiation with 3.5-Gy neutrons (□) or 10-Gy photons (●).

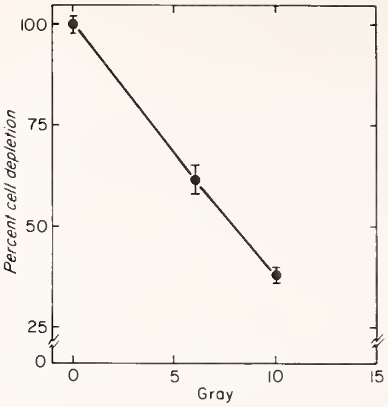


FIGURE 2.—Percent depletion of rat subependymal plate evaluated at 4 wk as function of photon dose. Points for 0 and 10 Gy have been replotted from fig. 1.

Cellular depletion as a function of time is presented in figure 1. Numerous pyknotic nuclei were noted when plates were examined on the day of irradiation. These changes were absent in sham-irradiated animals and after 1 week and subsequently in irradiated animals. There was no evidence of repopulation over the 10-week period, as has been previously described for this dose level (*12*). At 4 weeks following irradiation, there was no statistically significant difference in cellular depletion in photon- and neutron-irradiated animals; the RBE was 2.9.

Figure 2 presents the results of irradiating the subependymal plate with 6-Gy photons and evaluating cellular depletion after 4 weeks. Points for 0 and 10 Gy are redrawn from figure 1. Over this dose range, cellular depletion approaches linearity. A decision was made to evaluate subependymal plates in protection studies 4 weeks after irradiation.

Those studies involving photons were carried out in groups of 10 animals. Three test groups received γOH (Sigma, 20% weight/vol) ip at a dose of 0.5, 1.0, or 1.5 g/kg 30 minutes before receiving 10-Gy photons. At these doses, γOH is anesthetic and achieved animal restraint without the addition of chloral hydrate. One control group received γOH at 1.0 g/kg 30 minutes before sham irradiation and another control group received chloral hydrate at 0.3 g/kg before 10-Gy photons. In the neutron studies, 1 group of 5 animals received γOH at 1.0 g/kg before 3.5-Gy neutrons, and a control group of 5 animals received chloral hydrate at 0.3 g/kg before 3.5-Gy neutrons.

Results are shown in figure 3. Increasing protection is demonstrated with increasing doses of γOH. These differ-

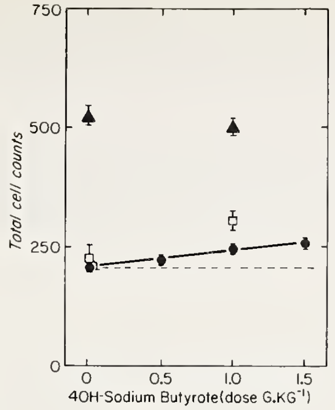


FIGURE 3.—Effect of γOH 30 min before irradiation on rat subependymal plate depletion evaluated 4 wk after sham irradiation (▲) or irradiation with 3.5-Gy neutrons (□) or 10-Gy photons (●).

ences reach statistical significance at ≥1.0 g/kg (photons, $P = .022$; neutrons, $P = .041$). By using the radiation dose/cellular depletion relationship in figure 2, the differences are calculated to be equivalent to a DRF of 1.05–1.22 for photons. In the absence of radiation, γOH did not significantly alter subependymal plate cellularity ($P = .32$).

Mean PaO_2 was 69.8 mm Hg in animals treated with chloral hydrate and 80 mm Hg in test animals receiving γOH at a dose of 1.0 g/kg. This difference was not significant ($P = .2$).

DISCUSSION

A previous study (*7*) indicated that γOH in anesthetic doses protected mice against TBI with a DRF of ~1.3. Unlike test animals, controls were not anesthetized, and radioprotection may have resulted from tissue hypoxia. In the present investigations, control animals were anesthetized. In addition, arterial blood gas analysis failed to demonstrate significant differences in PaO_2 between control and test animals. Nevertheless, the possibility remains that unrecorded differences in subependymal plate oxygen tension were present.

Classically, neuroglial turnover has been considered to be "slow." However, recent autoradiographic evidence suggests that, in adult mice at least, the subependymal plate neuroglial growth fraction is ~100 times that of cerebral endothelium (*9*). The early appearance of pyknosis in the subependymal plate after exposure to radiation, described >30 years ago (*18*) and confirmed in these experiments, is reminiscent of the rapid pyknotic response to radiation found in the crypts of Lieberkühn (*19*). An RBE of 2.9 for 15-MeV deuterons plus Be neutrons versus 250-kV (peak) x-rays is in agreement with the results of Chauser et al. (*12*).

Repair of DNA in vivo may be mediated by scavenging hydroxyl radical or $H^{\bullet}$ donation to sugar and base radical products. Sulfhydryl species, especially glutathione, are thought to be involved. It has been shown that γOH increases glucose flux through the pentose phosphate shunt in mammalian brain (*20*) and that, by providing NADPH, it may facilitate regeneration of reduced glutathione. The γOH is oxidized to succinic acid, which enters the TCA cycle (*21*). Should integrity of the TCA cycle be a prerequisite for γOH radioprotection, then protection may be selectively expressed in oxic normal cells since hypoxia and the malignant phenotype are associated with loss of TCA cycle activity.

In summary, this study demonstrates that γOH provides a small dose-dependent radioprotective effect on the rat subependymal plate when administered preceding single fractions of irradiation. It remains to be seen whether γOH similarly protects tumor or hypoxic tumor cells. Ultimately, an improvement in the therapeutic ratio may depend more on relative bioavailability and whether the effect of γOH is greater in a fractionated irradiation schedule.

REFERENCES

(*1*) NELSON DF, URTASUN RC, SAUNDERS WM, et al: Recent and current investigations of radiation therapy of malignant glioma. Semin Oncol 13:36–55, 1986.

(*2*) GERWECK LE, KORNBLITH PL, BURLETT P, et al: Radiation sensitivity of cultured human glioblastoma cells. Radiology 125:231–234, 1977.

(*3*) BLOOM HJG: Intracranial tumours: Response and resistance to therapeutic endeavours. Int J Radiat Oncol Biol Phys 8:1083–1113, 1982.

(*4*) WALKER MD, STRIKE TA, SHELINE GE: Dose-effect in radiotherapy of malignant gliomas. Int J Radiat Oncol Biol Phys 5:1729, 1979.

(*5*) STRONG AJ: γ-Hydroxybutyric acid and intracranial pressure. Lancet 1:1304, 1984.

(*6*) MAMELAK M, SCHARF BS, WOODS M: Treatment of narcolepsy with γ-hydroxybutyrate. A review of clinical and sleep laboratory findings. Sleep 9:285–289, 1986.

(*7*) DANA M, BARON C, LABORIT H: Action radioprotectrice du gamma-hydroxybutyrate de sodium. Agressologie 3:497–506, 1962.

(*8*) LEWIS PD: Mitotic activity in the primate subependymal layer and the genesis of gliomas. Nature 217:974–975, 1968.

(*9*) KORR H: Proliferation of different cell types in the brain. Adv Anat Embryol Cell Biol 61:32–33, 1980.

(*10*) HUBBARD BM, HOPEWELL JW: Quantitative changes in the cellularity of rat subependymal plate after X-irradiation. Cell Tissue Kinet 13:403–413, 1980.

(*11*) HOPEWELL JW, CAVANAGH JB: Effects of X-irradiation on the mitotic activity of the subependymal plate of rats. Br J Radiol 45:461–465, 1972.

(*12*) CHAUSER B, MORRIS C, FIELD SB, et al: The effects of fast neutrons and X-rays on the subependymal layer of the rat brain. Radiology 122:821–823, 1977.

(*13*) MORRIS AD, HOPEWELL JW: Combined effects of radiation and methotrexate on the cells of the rat subependymal plate. J R Soc Med 76:848–852, 1983.

(*14*) RUBINSTEIN LJ: Tumors of the central nervous system. *In* Atlas of Tumor Pathology, 2nd ser, fascicle 6. Washington, DC: US Armed Forces Institute of Pathology, 1972, pp 349–360.

(*15*) BOLDREY E, SHELINE G: Delayed transitory clinical manifestations after radiation treatment of intracranial tumors. Acta Radiol 5:5–10, 1966.

(*16*) ZEMAN W, INNES JRM: Craigie's Neuroanatomy of the Rat. London: Academic Press, 1963, p 219.

(*17*) WAYNFORTH HB: Experimental and Surgical Technique in the Rat. London: Academic Press, 1980, p 62.

(*18*) HICKS SP, MONTGOMERY PO: Effects of acute radiation on the adult mammalian central nervous system. Proc Soc Exp Biol Med 80:15–18, 1952.

(*19*) POTTEN CS: Extreme sensitivity of some intestinal crypt cells to X and γ irradiation. Nature 269:518–521, 1977.

(*20*) TABERNER PV, RICK JT, KERKUT GA: The action of gamma hydroxybutyric acid on cerebral glucose metabolism. J Neurochem 19:245–254, 1972.

(*21*) DOHERTY JD, ROTH RH: Metabolism of γ-[1-^{14}C] butyrate by rat brain: Relationship to the Krebs cycle and metabolic compartmentation of amino acids. J Neurochem 30:1305–1309, 1978.

WR-2721 as Cytotoxic and Radioprotective Agent in Treatment of Murine Lymphoma With Total Body Irradiation [1]

Lawrence R. Coia,* Darrell Q. Brown, and Janice Hardiman [2,3]

ABSTRACT—**The effectiveness of fractionated total body irradiation (TBI) in treatment of non-Hodgkin's lymphoma is limited by bone marrow toxicity. Because WR-2721 effectively protects bone marrow, we tested its potential in treatment of I-347 lymphoma in BALB/c mice, using various single and fractionated TBI regimens. In most treatment schedules, WR-2721 did not cause net lymphoma protection; in fact, it was cytotoxic. Lymphoma regrowth delay times for the 21 treatment groups were quite effectively fitted by a mathematical model with three components: 1) a dose-dependent radiation effect; 2) a small radioprotective effect by WR-2721; and 3) significant cytotoxicity of WR-2721. Bone marrow radioprotection was reduced when TBI was fractionated, but there was no evidence of WR-2721 cytotoxicity to marrow. The therapeutic gain due to WR-2721 was 2.5 for the five-fraction regimen, compared to 2.3 for a single fraction. The cumulative WR-2721 toxicity to lymphoma combined with marrow protection suggests that WR-2721 could increase the clinical therapeutic ratio of TBI, particularly fractionated TBI, in treatment of lymphoma.—NCI Monogr 6:235–239, 1988.**

In the management of patients with non-Hodgkin's lymphoma, TBI is effective as both primary and secondary therapy; however, bone marrow toxicity resulting in leukopenia and thrombocytopenia can be severe and is dose limiting (*1*). The radioprotective drug WR-2721 has been shown in laboratory animals to be an effective protector against the bone marrow injury caused by single-dose TBI (*2–4*). Significant differential radioprotection of bone marrow relative to lymphoma or cytotoxicity to lymphoma relative to bone marrow by WR-2721 could represent an important improvement in the treatment of lymphoma with TBI. The objective of this study was to determine in an animal lymphoma model with single-dose and fractionated TBI whether use of WR-2721 can result in a significant therapeutic gain and improvement in survival over use of TBI alone.

MATERIALS AND METHODS

Animal model.—A spontaneous thymus-derived lymphoma (I-347) described by Bosma et al. (*5*) was obtained from a mouse with SCID. This lymphoma was detected in approximately 20% of SCID mice autopsied at >4 months of age and was not seen in age-matched normal mice of the same inbred strain from which the SCID mice were derived. It grows initially as a solid T-cell lymphoma but then disseminates rapidly and shows a growth pattern and histology similar to that of human non-Hodgkin's lymphoma of the diffuse type.

Approximately 1-mm^3 fragments of this I-347 T-cell lymphoma were implanted sc in the dorsum of male BALB/c mice, which were subsequently randomized to 1 of several treatment groups when the tumors reached the desired size of 700–1,100 mm^3.

WR-2721 distribution.—The tissue distribution of ^{14}C-labeled WR-2721 was determined for a variety of normal tissues including plasma, liver, spleen, kidney, heart, and bone marrow and for this lymphoma model in BALB/c mice. Three mice, each bearing 1 tumor were killed (30 min following ip injection of ^{14}C-WR-2721 at 365 mg/kg), and the mean ^{14}C counts per minute for each normal tissue and tumor were determined.

Lymphoma response to TBI and WR-2721.—Lymphoma response to treatment was determined by tumor regrowth delay time (T_2). The six categories of treatment included a control; WR-2721 alone; single-dose irradiation alone; daily fractionated irradiation alone; single-dose irradiation plus WR-2721; and daily fractionated irradiation plus WR-2721. There were 21 treatment groups with 10 mice treated in each group (table 1). Mice receiving WR-2721 were given one-half of the maximum tolerated dose (365 mg/kg ip) alone or 30 minutes prior to TBI. The control mice received 0.5 ml of saline ip. The TBI was carried out with a ^{137}Cs-γ Shepherd Model 280 irradiator at 198 cGy/minute. During irradiation, each mouse was enclosed in a heavy Lucite cylinder supplied with breathing air. Tumors were measured every other day with a vernier caliper and annular thickness gauges. Tumor volumes were calculated as the product of three perpendicular dimensions times $\pi/6$ since they were approximately ellipsoid in shape. The individual T_2 values were determined by interpolation of tumor volume data at twice treatment volume. Group means of these individual T_2 values were then calculated for each

---

ABBREVIATIONS: TBI = total body irradiation; SCID = severe combined immunodeficiency; T_2 = tumor regrowth delay time; DRF = dose reduction factor; $LD_{50/30}$ = lethal dose for 50% of mice at 30 days; TG = therapeutic gain(s).

[1] Supported by Public Health Service grants CA-37921 and CA-06927 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Department of Radiation Therapy (*L. R. Coia, D. Q. Brown,* and *J. Hardiman*), Fox Chase Cancer Center, and Department of Radiation Therapy (*L. R. Coia* and *D. Q. Brown*), University of Pennsylvania School of Medicine, Philadelphia.

[3] We thank Dr. Gerald Hanks for assistance in reviewing the manuscript; Dr. Samuel Litwin for statistical analysis; and Mary Davis, Jeffrey Gold, Richard Pogach, David Mann, John Pittock, and Christine Webb for technical assistance.

* *Reprint requests:* Lawrence R. Coia, M.D., Department of Radiation Therapy, Fox Chase Cancer Center, Central and Shelmire Aves., Philadelphia, PA 19111.

Table 1.—Experimental and theoretical lymphoma regrowth delay times (T_2) associated with various WR-2721 and fractionated TBI combinations[a]

Treatment (No. of fractions × cGy)	Experimental T_2 (mean ± SE in hr)	Model T_2[b] (hr)
Saline		
1×0	62 ± 10	63
1×40	74 ± 5	67
2×40	76 ± 7	72
3×40	78 ± 10	76
5×40	84 ± 11	86
1×80	78 ± 6	81
1×120	90 ± 9	99
1×200	141 ± 9	145
1×400	393 ± 75	381
WR-2721		
1×0	76 ± 9	73
2×0	84 ± 7	84
3×0	95 ± 9	97
5×0	132 ± 14	129
1×40	66 ± 9	77
2×40	103 ± 6	93
3×40	109 ± 11	113
5×40	166 ± 22	167
1×80	87 ± 7	90
1×120	103 ± 10	105
1×200	162 ± 20	145
1×400	311 ± 41	322

[a] WR-2721 (365 mg/kg) or saline was given ip 30 min prior to each daily fraction of TBI.

[b] *See* text.

dose level of interest. The DRF values were determined for single-dose and fractionated treatment as the ratio of daily TBI doses given with WR-2721 divided by the daily dose of TBI alone, which results in the same doubling time.

Bone marrow response to TBI and WR-2721.—Bone marrow response to treatment was determined by the $LD_{50/30}$ method. Three treatment categories included fractionated TBI alone; fractionated TBI preceded by WR-2721 to determine radiation protection; and fractionated TBI followed by WR-2721 to determine if WR-2721 bone marrow cytotoxicity was present. The WR-2721 was given ip at 365 mg/kg 30 minutes prior to or 1 hour following irradiation. The TBI using the ^{137}Cs-γ irradiator was given in five constant daily fractions of 170–410 cGy per fraction. Mice were followed and scored for survival. Then the $LD_{50/30}$ for each treatment group was determined by probit analysis (*6*). The DRF was computed as the ratio of the $LD_{50/30}$ of fractionated TBI plus WR-2721 divided by the $LD_{50/30}$ of fractionated TBI alone.

Effect on survival.—To determine whether addition of WR-2721 to TBI treatment increases the overall mouse survival at these low noncurative radiation dose levels, we treated 2 groups of lymphoma-bearing mice with five daily 40-cGy fractions of TBI. At 30 minutes before each TBI fraction, we administered either WR-2721 at a dose of 365 mg/kg or 0.4 ml of saline ip. Mice were observed daily for survival, and differences in the survival means were compared by the Student's *t*-test.

Lymphoma cell lethality of WR-2721.—To determine whether WR-2721 toxicity to the lymphoma involved a tumor cell lethality rather than just a sublethal toxicity, either of which could result in prolongation of doubling time, we examined the trypan blue exclusion of tumor cells from WR-2721-treated mice, compared with that for control mice. At 2, 3, or 4 hours following ip injection of WR-2721 at 365 mg/kg, mice were killed and the tumors were excised, minced, and filtered through a No. 100 stainless-steel mesh. After centrifugation and resuspension in medium containing 0.04% trypan blue, the percent viability (i.e., dye exclusion) of single tumor cells was determined using a hemacytometer. Differences in viability were compared by the Student's *t*-test.

RESULTS

Analysis of ^{14}C-WR-2721 tissue distribution indicates a significant concentration of the radioprotector in several organs as well as in the lymphoma (table 2). Uptake measured 30 minutes after ip injection of labeled WR-2721 was greatest in kidney and liver. Lymphoma uptake was 45% that of kidney, while bone marrow uptake was substantial at 534 disintegrations/minute per 10^6 cells and was similar to that of lymphoma.

The T_2 values resulting from various single or fractionated treatments with or without WR-2721 are listed in table 1. These data suggest that T_2 is dependent on total radiation dose and total WR-2721 dose. Comparison of the T_2 for single-dose and fractionated irradiation alone and WR-2721 alone indicates that the magnitude of the WR-2721 effect at doses of 365 mg/kg is slightly greater than the effect of TBI at 40-cGy fractions. It also appears that there is little radioprotective effect of WR-2721 and that fractionating the irradiation diminishes the T_2 value.

A theoretical model was developed, which takes into account these effects. The equation for the model is:

$$\ln T_2 = \frac{Anx(d - D_q)}{(1 + Ew)} + Bm + C,$$

Table 2.—Distribution of ^{14}C-WR-2721 in lymphoma-bearing mice 30 min after injection of WR-2721[a]

Mouse No.	Tumor	Plasma	Liver	Kidney	Spleen	Heart	Bone marrow
1	371	317	1,226	1,102	869	762	486
2	574	310	1,212	1,079	838	733	586
3	547	318	1,129	1,096	796	747	531
Mean	497	315	1,189	1,092	834	747	534
± SE	70	5	36	21	23	19	29

[a] WR-2721 was given at a dose of 365 mg/kg ip. Values = disintegrations/min per mg except for distribution in bone marrow, which is given in disintegrations/min per 10^6 cells.

where the treatment parameters are:

n = number of radiation fractions;
x = 1 when d is not zero; $x = 0$ when $d = 0$;
d = daily radiation dose;
m = number of doses of protector; and
w = 1 if protector was given and 0 if it was not given.

The model constants, which were optimally fitted to the data using a modified form of multiple linear regression analysis, were:

A = radiation effectiveness coefficient $= 4.8185 \times 10^{-3}$/cGy;
B = radioprotector cytotoxicity coefficient $= 0.1432$;
C = additive constant $= 4.1443$;
D_q = radiation quasithreshold dose $= 26.95$ cGy; and
E = radioprotection increment coefficient $= 0.210$.

In the first term on the right side of this equation, the daily radiation dose is reduced by the quasithreshold dose, multiplied by the number of radiation fractions, and divided by a quantity resembling a protection factor or DRF. The second term describes the WR-2721 cytotoxicity to lymphoma cells. The T_2 values computed by this model are listed in table 1 and compared with the 21 experimental values. Note that 18 of these computed values lie within the SE of the experimental values. These effects on tumor regrowth of WR-2721 with single-dose and fractionated TBI are illustrated in figures 1 and 2, respectively. The T_2 values determined experimentally along with their associated SE are plotted for each single and fractionated TBI dose given. The lines drawn in each figure were generated by the theoretical model as fitted to the experimental data. The effect of WR-2721 with single doses of radiation is minimal (fig. 1). The DRF for a single 200-cGy fraction equals 1.0. However, with fractionated irradiation, the addition of WR-2721 demonstrates a significant increase in T_2 due to its cumulative cytotoxicity. The DRF is therefore <1 for fractionated treatment; for the five-fraction 40-cGy treatment, the DRF was determined to be 0.59. By using the model, the dose of TBI in five daily fractions plus saline, which results in the same doubling time as five daily fractions of 40 cGy + WR-2721, can be found to be 67.5 cGy, so DRF $= 40/67.5 = 0.59$.

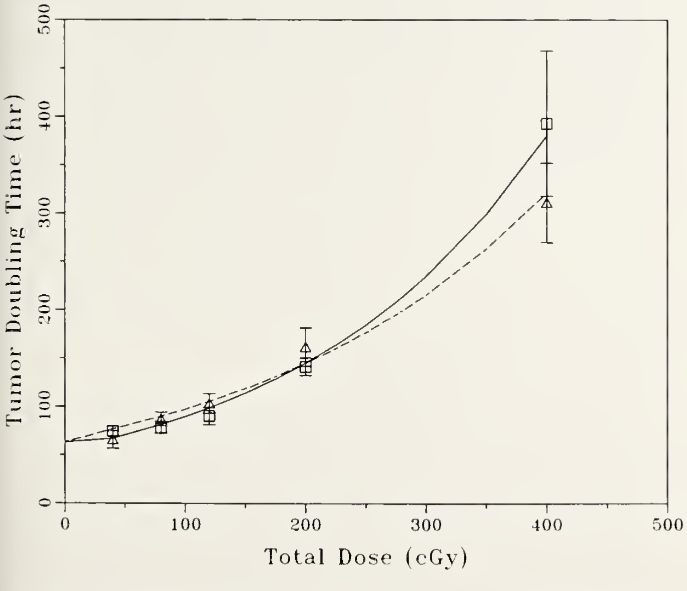


FIGURE 1.—Lymphoma regrowth delay time expressed as volume doubling time (T_2) for various single TBI doses. WR-2721 administered 30 min prior to TBI (△) had very little effect with this single-fraction treatment, compared with controls (□), which received saline 30 min prior to TBI. *Error bars* indicate SE. *Lines* represent fitted mathematical model described in text for WR-2721 (*dashed*) and saline (*solid*).

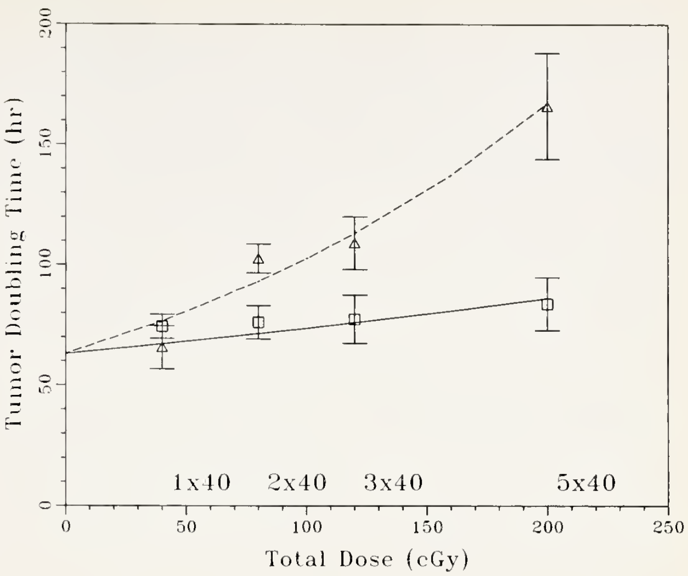


FIGURE 2.—Lymphoma regrowth delay time expressed as volume doubling time (T_2) for various fractionated TBI doses. WR-2721 administered 30 min prior to each daily 40-cGy fraction (△) of TBI had cytotoxic effect, compared with controls (□), which received saline 30 min prior to each fraction. *Error bars* indicate SE. *Lines* represent fitted mathematical model described in text for WR-2721 (*dashed*) and saline (*solid*).

The effects on bone marrow as measured by the $LD_{50/30}$ are presented in table 3. There is an increase in the $LD_{50/30}$ with fractionated treatment, compared to large single doses of radiation. With WR-2721 preceding irradiation, the computed DRF for five-fraction TBI is 1.45, compared to

TABLE 3.—Effect of fractionation on WR-2721 radioprotection of bone marrow using $LD_{50/30}$ end point for TBI[a]

Treatment	Protection	$LD_{50/30}$ (cGy)[b]	DRF
Single dose[c]	None	960 ± 12	2.29
	WR-2721 at 30 min before TBI	2,195 ± 24	
Fractionated			
5 daily fractions	None	1,133 ± 35	1.45
	WR-2721 at 30 min before TBI	1,639 ± 34	
5 daily fractions	None	1,133 ± 35	1.00
	WR-2721 at 60 min after TBI	1,131 ± 34	

[a] WR-2721 was given at dose of 365 mg/kg ip.

[b] Values = mean ± SE.

[c] Single-dose data were determined in this laboratory in prior experiment (*4*).

2.29 for large single-dose treatment. There is no evidence that WR-2721 given 1 hour following TBI is cytotoxic to bone marrow since the $LD_{50/30}$ is not significantly changed.

The TG was defined as the ratio of the DRF for bone marrow to the DRF for lymphoma. The TG is better for fractionated treatment than for single-dose treatment. The 200-cGy single-dose DRF for lymphoma compared to the single-dose DRF for bone marrow yields a TG of 2.3, while the DRF for lymphoma treatment with five 40-cGy fractions compared to the DRF for five-fraction bone marrow treatment results in a TG of 2.5. However, different dose ranges were necessarily utilized in determining the DRF values for the marrow and lymphoma end points.

A small but significant improvement in survival was seen even with a noncurative fractionated TBI schedule. When WR-2721 was added to five daily fractions of 40-cGy TBI, the survival of lymphoma-bearing mice was increased from 25.0 ± 0.5 days (mean ± SE; $N=10$) to 27.3 ± 0.8 days ($N=11$). This difference is statistically significant (Student's *t*-test, $P \leq .02$).

It appears that direct lymphoma cell lethality does result from WR-2721 administration, as tumor cell viability determined by trypan blue exclusion was reduced from 88 ± 4% ($N=10$) in control tumors to 71 ± 5% ($N=10$) in tumors from WR-2721-treated mice. This difference is statistically significant (Student's *t*-test; $P \leq .01$) and did not appear to depend on time (2, 3, or 4 hr) after WR-2721 injection.

DISCUSSION

Our findings of an increased lymphoma regrowth delay due to WR-2721, significant bone marrow protection by WR-2721, and improved survival all support its potential use in the treatment of lymphoma with fractionated TBI. The DRF values obtained for WR-2721 resulted in relatively large TG of 2.3 and 2.5 for single-dose and fractionated TBI, respectively. Due predominantly to the lack of lymphoma radioprotection and to the cumulative cytotoxicity of WR-2721 on the lymphoma, we observed a decrease in the lymphoma DRF with fractionated treatment, compared to single-dose treatment. We also observed a decrease in bone marrow DRF with fractionation. Travis et al. (*7*) have also reported a similar decrease in bone marrow DRF for WR-2721 (200 mg/kg) from 2.3 for single-fraction irradiation to 1.10 with a four-fraction regimen. They did not observe any direct bone marrow cytotoxicity of WR-2721, using a spleen colony assay.

This particular lymphoma was chosen because it exhibited a growth and radiation response pattern similar to that of many human non-Hodgkin's lymphomas. Although it grows with a local solid component phase, it also demonstrates rapid development of distant metastases without a significant leukemic phase (*5*). Many available leukemias/lymphomas can be grown as ascites but not as an easily measurable solid tumor. The attributes of both solid tumor growth and metastatic presence can affect the uptake and protection of WR-2721. Milas et al. (*8*) have shown that the degree of protection of WR-2721 varies with tumor size; for a given tumor, microaggregates are better protected than larger cell masses. We found local tumor uptake of WR-2721 to be approximately the same as that of bone marrow, though protection was much less. The readily quantifiable response of the local tumor to low doses of radiation and to WR-2721 allowed the formulation of a useful predictive mathematical model for regrowth delay. Survival, which may have been affected by distant disease, was significantly improved by use of WR-2721 with TBI.

Direct cytotoxicity of WR-2721 as well as potentiation of chemotherapeutic cytotoxicity by WR-2721 have been observed in the laboratory and suggested in clinical trials. Meistrich et al. (*9*) reported direct cytotoxicity on mouse testicular stem cells, and Rojas and Stewart (unpublished data, 1983) observed skin toxicity by WR-2721 under certain circumstances. Valeriote and Grates (*10*) reported a potentiation of cytotoxicity of mechlorethamine by WR-2721 against a transplanted leukemia. Yuhas (*11*) observed a toxic effect by WR-2721 on cells involved in the T-cell arm of the immune response; however, this effect on T-cells appeared to be outweighed by the radioprotective effect. Radioprotection of immunologically reactive T-lymphocytes was subsequently corroborated by Harris and Menese (*12*). Early clinical observations by Glover et al. (*13*) suggest that WR-2721 may potentiate the antitumor activity of cisplatin on melanoma and squamous cell carcinoma of the head and neck. We have not demonstrated the mechanism of cytotoxicity of WR-2721 to this lymphoma, since proof of tumor cell lethality by trypan blue exclusion is crude and needs to be corroborated by clonogenic assay. Nonetheless, we have observed an increase in tumor growth delay by WR-2721 of a similar magnitude to the effect of TBI at 40 cGy, and the effect is cumulative. This effect of WR-2721 alone has not been previously reported in any tumor system.

Controversy exists as to whether DRF values for WR-2721 in tumors are comparable to those obtained in normal tissues. There is additional concern that most studies of radiation protection have been carried out with large single doses of radiation, so it is uncertain whether the small DRF values obtained for tumor under these conditions are maintained with small fractionated doses like those used in conventional radiation therapy. Rojas et al. (*14*) recently addressed these points. They reported a similar degree of protection for normal tissues (skin and kidney) and tumors, and this protection did not change significantly with fractionation. The dilemma that must be addressed before clinical application of these findings in phase I–II trials is whether to give WR-2721 with standard total radiation doses and fractionation, with the risk of underdosing the tumor due to radioprotection, or to escalate the total radiation dose in the absence of clinical evidence of protection, thereby risking complications.

The five-fraction regimen utilized in this experiment was designed to be similar to a known effective clinical regimen for non-Hodgkin's lymphoma, which utilizes five fractions of low-dose (15-cGy) radiation twice weekly to a total dose of 75 cGy (*15*). This dose schedule is then repeated following hematologic recovery approximately 1 month later. It is not known whether TBI can be repeated at relatively close intervals, although Rostom and Peckham (*16*) report success with 10 fractions of 15 cGy over 12 days. Clinical use of WR-2721 with fractionated TBI may allow a larger total dose of TBI to be delivered or may result in less severe hematologic depression with a standard dose.

REFERENCES

(*1*) Chaffey JT, Hellman S, Rosenthal DS, et al: Total-body irradiation in the treatment of lymphocytic lymphoma. Cancer Treat Rep 61:1149–1152, 1977.

(*2*) Yuhas JM, Storer JB: Chemoprevention against three modes of radiation death in the mouse. Int J Radiat Biol 15:233–237, 1969.

(*3*) Rasey JS, Nelson NJ, Mahler P, et al: Radioprotection of normal tissues against gamma rays and cyclotron neutrons with WR-2721: LD50 studies and ^{35}S-WR-2721 biodistribution. Radiat Res 97:598–607, 1984.

(*4*) Brown DQ, Pittock JW, Rubinstein JS: Early results of the screening program for radioprotectors. Int J Radiat Oncol Biol Phys 8:565–570, 1982.

(*5*) Bosma GC, Custer RP, Bosma MJ: A severe combined immunodeficiency mutation in the mouse. Nature 301: 527–530, 1983.

(*6*) Finney DJ: Probit Analysis, 2nd ed. Cambridge, England: Cambridge Univ Press, 1962.

(*7*) Travis E, Thames H, Tucker S, et al: Protection of mouse jejunal crypt cells by WR-2721 after small doses of radiation. Int J Radiat Oncol Biol Phys 12:807–814, 1986.

(*8*) Milas L, Hunte N, Ito I, et al: Effect of tumor type, size, and endpoint on tumor radioprotection by WR-2721. Int J Radiat Oncol Biol Phys 10:41–48, 1984.

(*9*) Meistrich ML, Finch BS, Hunter N, et al: Protection of spermatogonial survival and testicular function by WR-2721 against high and low doses of radiation. Int J Radiat Oncol Biol Phys 10:2099–2107, 1984.

(*10*) Valeriote F, Grates HE: Dose and interval relationship for the interaction of WR-2721 and nitrogen mustard with normal and malignant cells. Int J Radiat Oncol Biol Phys 10:1561–1564, 1984.

(*11*) Yuhas JM: Radioprotective and toxic effects of S-2-(3-amino-propylamino)ethylphosphorothioic acid (WR-2721) on the development of immunocompetent cells. Cell Immunol 4:256–263, 1972.

(*12*) Harris JW, Menese JJ: Radioprotection of immunologically reactive T lymphocyte by WR-2721. Int J Radiat Oncol Biol Phys 4:437–440, 1978.

(*13*) Glover D, Glick JH, Weiler C, et al: Phase I trials of WR-2721 and *cis*-platinum. Int J Radiat Oncol Biol Phys 10:1781–1784, 1984.

(*14*) Rojas A, Stewart FA, Soranson KA, et al: Fractionation studies with WR-2721: Normal tissues and tumour. Radiother Oncol 6:51–60, 1986.

(*15*) Carabell S, Chaffey JT, Rosenthal DS, et al: Results of total body irradiation in the treatment of advanced non-Hodgkin's lymphoma. Cancer 43:994–1000, 1979.

(*16*) Rostom AY, Peckham MJ: Total body irradiation in advanced non-Hodgkin's lymphoma. Eur J Cancer 13: 1241–1249, 1977.

Discussion Summary

Robert F. Ozols[1] and G. Gordon Steel[2,*]

The varied series of posters in this session were grouped under five headings:

INTERACTIONS BETWEEN RADIATION AND CYTOTOXIC DRUGS

The discussion participants sought at the outset to clarify definitions of radiation resistance and drug resistance. When patients are found to be less responsive to radiation or drugs subsequent to a course of treatment, this response may result from various processes apart from induced cellular resistance. There may be cell kinetic and vascular changes and also a tendency for such patients to have more advanced and perhaps more aggressive disease than would have been the case on presentation. We need cell survival curves to demonstrate true cellular radioresistance or chemoresistance; the extent of increased resistance should be expressed not as a ratio of surviving fractions at a fixed dose but as a ratio of doses required to produce a particular level of effect.

The induction of radioresistance by repeated or prolonged irradiation is a rare phenomenon in mammalian cells. When this has been observed, it has occurred after the accumulation of large total doses of continuous irradiation. The induction of radioresistance by exposure to cytotoxic drugs is also rare. Induced drug resistance often results from impaired drug access into tumor cells. Cross-resistance with radiation most likely may occur when drugs act at the DNA level by mechanisms closely similar to radiation, i.e., by free radical production.

Pleiotropie drug-resistant cell lines sometimes show high levels of resistance to drugs. Doubt was cast on whether such levels of resistance are really observed clinically.

NONCYTOTOXIC DRUGS THAT MODIFY RADIOSENSITIVITY

There is considerable interest in the use of agents that inhibit the repair of radiation damage in tumor cells. It is clear that because the normal tissues included in the radiation field depend on repair processes for their tolerance of radiation, we require repair inhibitors that show some degree of tumor selectivity. There was speculation that genes could be identified whose amplification is responsible for tumor cell resistance and then specific inhibitors of these genes be developed. Examples were given of drugs, such as cisplatin, that enhance tumor cell response to radiation without leading to enhancement of normal tissue damage. One such example is the failure of cisplatin to enhance damage to the mouse kidney, as was recently demonstrated by Dr. Fiona Stewart. When treatment is limited by late normal tissue reactions, it is possible therefore for one to find drugs that do not seriously enhance damage when given concurrently with radiation.

At the present time, we have insufficient information on the kinetics of repair of radiation damage in tumor cells and normal tissue stem cells. If, in certain therapeutic situations, considerable differences in repair half-times are observed between tumor and normal cells, then this might lead to ways of exploiting the kinetics of repair with suitable timing of treatment.

There was some discussion of radioprotection when WR-2721 is used. In evaluating the effects of this agent, it is important that we avoid gross interference with metabolism and the lowering of body temperature. At least one of the metabolic products of WR-2721 reacts rapidly with oxygen and could conceivably create hypoxic conditions in vivo. Peroxide may be produced, which may require that catalase be given concurrently with the radioprotector.

ROLE OF GLUTATHIONE

The complexity of the role of glutathione in vivo was strongly emphasized. Although simple mechanisms for its involvement in free radical processes have been described, it is most unlikely that these will occur in isolation. It was surmised that there may be dozens of enzymes that are inhibited when glutathione levels are artificially driven down, when, for instance, buthionine sulfoximine is used. There is some evidence that modification of glutathione levels has little effect on the aerobic radiation response, but it was pointed out that, in very well-defined cell culture systems in which the thiol content of the medium has been carefully reduced, a substantial enhancement of radiation response then results from glutathione depletion. Even then, you may have to deplete glutathione to around one-millionth of its normal level to obtain a good degree of sensitization. Perhaps one-third of the enzymes in the cell are sulfhydryl dependent in one way or another, and it is impossible for one to know which of these enzymes are going to be the most important in a particular situation.

Some discussion ensued about the predictive importance of glutathione levels in human cell lines. It has been suggested that high glutathione competes with misonidazole and thus reduces the effectiveness of this radiosensitizer.

---

[1] Experimental Therapeutics Section, Medicine Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bldg. 10, Rm. 12N226, National Institutes of Health, Bethesda, MD 20892.

[2] Radiotherapy Research Unit, Institute of Cancer Research, Royal Cancer Hospital, Clifton Ave., Sutton, Surrey SM2 5PX, United Kingdom.

[*] *Reprint requests:* G. Gordon Steel, M.D., Radiotherapy Research Unit, Institute of Cancer Research, Royal Cancer Hospital, Clifton Ave., Sutton, Surrey SM2 5PX, United Kingdom.

How relevant are the enzyme assays on in vitro cell lines? A variety of possible artifacts were mentioned. Glutathione levels vary during the growth period of cultures and tend to be maximal during logarithmic phase. Because investigators tend to believe that the cells are healthiest when in logarithmic phase, there may have been a tendency to overestimate the glutathione levels that might arise in vivo. Glutathione levels vary through the cell cycle, and further research on this is needed, perhaps with flow cytometry. Evidence from the University of Rochester (NY) Cancer Center has shown that glutathione levels are unaffected by elutriation at 4° C. Further work from the same laboratory has shown that the glutathione levels in cell cultures are threefold to fivefold higher than in the tumors from which they were derived. Human lung and colon tumors freshly explanted into tissue culture also give lower glutathione levels than in established cell lines that result from them. It was pointed out that these results might arise from inappropriate levels of amino acids or glucose in the culture medium.

The response of cells to glutathione depletion may depend not only on the absolute level of suppression achieved but also on the kinetics of its rebound. Cells may differ in the speed with which they recover from a particular level of glutathione depletion.

MECHANISMS OF RADIORESISTANCE AND CHEMORESISTANCE

The in vivo response of tumors to radiation could be said to be the composite result of inherent cellular radiosensitivity, modified by factors in the environment of tumor cells. The work of Fertil and Malaise in Paris and more recently that by Deacon et al. in London has shown that, among a variety of human tumor types, the correlation is significant between the initial slope of the oxic radiation cell survival curve and curability from clinical radiotherapy. This leads to the view that inherent cellular radiosensitivity is at least a significant factor in clinical response, a conclusion that is supported by the work of Weichselbaum. The differences that have been found so far are between tumors of various histologic types. Are there also differences within a tumor type? So far as we know, there is no strong evidence for this and it needs careful researching.

The use of low-dose-rate irradiation to identify more clearly the initial slope of the cell survival curve is described by the poster of Kelland et al. In discussion, we learned that data are accumulating on the possible therapeutic advantages of low-dose-rate irradiation. These data show that the amount of sparing observed varies widely among human tumor cells and covers a similar range to the sparing seen in various normal tissues. In a particular clinical situation, therefore, one could have either a therapeutic benefit (little sparing of tumor cells but substantial sparing of normal tissues) or a therapeutic disadvantage (sparing of tumor cells coupled with little sparing of normal tissue damage). This is an area of study in which predictive assays might offer considerable advantages.

The determinants of inherent cellular radiosensitivity were considered. The lethal/potentially lethal model of Curtis allows a description of dose-rate-dependent cellular radiosensitivity in the probability of producing lethal or potentially lethal lesions within cells, coupled with the relative rate constants for repair and misrepair of potentially lethal lesions. Researchers' attempts to describe differences in radiosensitivity among human tumor cells of this model are to some extent frustrated by its flexibility because it is often difficult to obtain sufficiently full and precise data to define accurately the four parameters of Curtis' model.

The work of Lehnert presented here has stressed the factors other than intrinsic radioresistance that are important in the radioresponsiveness of human tumor xenografts. Of course, the principal environmental determinant of radioresistance is hypoxia. In addition to cells that are chronically and deeply hypoxic, there may well be a substantial proportion of tumor cells that have intermediate sensitivity or whose hypoxic status varies transiently. In Lehnert's work, the dose rate was about 2 Gy/minute, and it took about 5 minutes to deliver 10 Gy. Therefore, transient changes would have to occur over this sort of time span to have influenced the results that she obtained. Reference was made to the work of Chaplin and co-workers in Vancouver, who demonstrated both acutely and chronically hypoxic cells within experimental tumors and found that the acutely hypoxic cells change their sensitivity over periods of approximately 10–20 minutes.

The selective killing of hypoxic cells was addressed from the point of view of oxygen metabolism. Tumor cells become hypoxic because of the depletion of oxygen by their own metabolism. The major substrates are glutamine, glutamate, and glucose; we know that interstitial fluid is low in glucose. Therefore, there is a case for our trying to exploit the metabolic properties of tumors, perhaps by giving glucose or glutamate in conjunction with drugs that are intended to kill hypoxic cells selectively.

DNA STRUCTURE, GENE AMPLIFICATION, AND IDENTIFICATION OF REPAIR GENES

Interest is growing in the extent to which the infliction and repair of radiation damage to DNA is dependent upon the compactness of chromatin. The work of Oleinick and others has shown considerable differences in radiosensitivity between transcribing and nontranscribing regions of DNA. Over the past few years, considerable progress has been made by investigators in identifying genes that have a role in determining radiation sensitivity. Gene amplification has so far been demonstrated only in tumor cells. Participants in this discussion were not aware of any reports of this process occurring in the stem cells of normal tissues. One of the exciting possibilities that the gene amplification story brings up is predictive testing based not on our looking at drug sensitivity but actually investigating specific amplified genes. If mechanisms of resistance can be clarified, we might derive molecular probes to specific amplified genes and attempt to overcome resistance in a way that is based on such identification rather than on purely empirical drug sensitivity testing. It may well be that there are multiple mechanisms of resistance that may be expressed within a tumor cell population. Thus one may have to use a whole cocktail of agents to circumvent the different mechanisms of resistance.

At this meeting, a report was given on the induction of gene amplification by transient hypoxia. The extent to which this might occur in tumors in vivo may be small, but Rice commented that one only needs to have gene amplification in a small proportion of tumor cells for this subpopulation eventually to grow out and dominate the response to chemotherapy. The relative uncommonness of induced radioresistance could be due to the requirement for a large bank of genes to be amplified before a significant change in response occurs.

Attention was drawn to stress proteins induced by hypoxia and their possible influence on drug resistance. It is possible that oxygen-regulated proteins are the products of amplified genes; we need to understand more clearly the role of these proteins in the in vivo appearance of drug resistance.

The development of drug resistance in small cell lung cancer was cited as a case in which it is important that intracellular changes be distinguished from the effects of cell selection. Induced drug resistance is sometimes associated with the appearance of large cell or squamous cell carcinoma variants. Some think that when this occurs it reflects the heterogeneity of the original tumor cell population and the selective breeding out of cell lines that are resistant to drug treatment. This led to a discusson on whether resistant genes preexist in untreated tumor cell populations or are generated as a result of drug treatment. Participants thought that probably both situations occur.

Combined Modality Treatment—I

Keynote Address: Combined Modality Treatment for Primary Tumor

Harry Bartelink[1,*]

ABSTRACT—The combination of radiotherapy and chemotherapy is aimed at improving local control and eradicating distant metastases by using cytostatic drugs as radiosensitizing or independent cell-killing agents. This approach has been successful in some areas but has failed in a large number of trials. The failure can be attributed to biologic and clinical factors; the low drug uptake in the tumor is one of the most limiting factors. New approaches should therefore include the improvement of drug targeting and the use of predictive assays; these efforts should proceed with the performance of pharmacokinetic studies and experiments testing the effects of combined treatment on tumors and normal tissues. Pharmacokinetic studies should include measurement of the intracellular drug uptake, preferably at the target. This is now possible for cisplatin; DNA adducts induced by this drug can now be measured with an immunocytochemical technique. The experimental animal studies should include testing of the effects of fractionated irradiation with drugs on tumors and on early- and late-responding normal tissues.—NCI Monogr 6:247–251, 1988.

The need to improve the efficacy of radiotherapy alone in controlling primary tumors has led to widespread use of the combination of radiotherapy and chemotherapy. The main aims of using this combined approach are to improve local control and to sterilize distant metastases. Before this combined approach can be accepted as standard therapy for optimal local control of the primary tumor, the following points should be considered:

1) Does an increased local tumor control rate lead to an improved survival rate and a better quality of life?
2) Does evidence exist that this combined approach has led to better local control and survival rates?
3) Why have many trials failed to show an improvement?
4) Are there ways to improve the combined treatment in the future to obtain higher local control rates?
5) What are the criteria to select patients, drugs, and treatment schedules for this combined treatment?

IMPORTANCE OF IMPROVED LOCAL CONTROL

Does an increased local tumor control rate lead to an improved survival rate and a better quality of life, or will the advantage of achieving a decrease in recurrences be lost because of an increase in distant metastases as a result of longer life expectancy or because of an increase in the complication rate?

It is obvious that an absolute prerequisite for cure of a cancer patient is the eradication of the primary and regional disease. An improved survival rate can therefore be expected if more patients are successfully treated for the primary lesion, besides the decrease of patients dying with uncontrollable local symptoms. At the meeting in Rome, Italy, in 1985 (The Challenge of Local Tumor Control and Its Impact on Survival), Suit and Westgate (*1*) demonstrated that when either higher-dose radiation or a combination of radiation with other treatment modalities was administered, a better survival was achieved in patients with medulloblastoma or tumors of the head and neck area, prostate, rectum, or bladder. Despite these achievements, further improvement in local control after irradiation alone is especially necessary for tumor sites associated with a poor prognosis and death from uncontrolled local disease; e.g., malignant tumors of the head and neck area, cervix, and bladder (*2*). In dying patients, better local control will prevent such symptoms as ulceration or pain caused by tumor involvement in nerves. More effective local treatment with radiation may also avoid multilating surgery, especially in the head and neck or pelvic areas.

An effective way to improve local control is to administer higher radiation doses. For example, Bartelink et al. (*3*) showed that increasing the total radiation dose resulted in a decrease in local recurrences in patients with nodal metastases from squamous cell carcinoma in the head and neck region. This same relationship remained after adjustment for other prognostic factors such as tumor volume.

The gain in achieving a higher local control rate disappeared, however, at doses >70 Gy in 7 weeks, due to a sharp increase in serious late complications in normal tissue (fig. 1). Methods other than application of higher radiation doses should therefore be explored to improve the efficacy of irradiation without increasing the late radiation damage.

IMPROVED LOCAL CONTROL AND SURVIVAL

The combination of radiotherapy and chemotherapy has often been used during the last two decades to achieve the aims of greater local control and higher survival rates. Nevertheless, evidence that this effort has been successful is limited to a small number of trials (*4*). Examples of improved local control and survival confirmed in randomized trials are the use of 5-fluorouracil for oral cavity tumors (*5*), lomustine for brain tumors (*6*), and bleomycin for head and neck tumors (*7,8*). Two other trials using

---

ABBREVIATIONS: EORTC = European Organization for Research and Treatment of Cancer; DMF = dose-modification factor(s).

---

[1] Department of Radiotherapy, The Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Amsterdam.

[*] *Reprint requests:* Harry Bartelink, M.D., Department of Radiotherapy, The Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

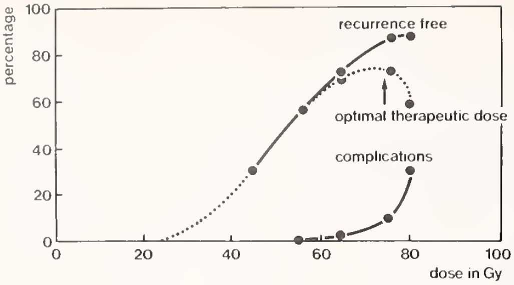


FIGURE 1.—Relation of radiation dose (in Gy) to recurrence percentages in irradiated part of neck and to complication rate. Optimal therapeutic dose was 70 Gy. Modified with permission from (*3*).

bleomycin with irradiation in head and neck tumors, however, did not confirm these results (*9,10*). Extensive reviews of the literature by Carter (*11*) and Tannock and Browman (*12*) reveal that no improvement in survival is achieved by the combination of radiotherapy and chemotherapy in most randomized trials.

A more promising area for combined modality treatment may be the avoidance of mutilating surgery in patients with head and neck or pelvic tumors. For example, the excellent local control rate with radiotherapy combined concomitantly with 5-fluorouracil and mitomycin avoids the need for colostomy in the majority of patients with anal cancer (fig. 2) (*13,14*). Because these results have not been compared with those of irradiation alone in a randomized trial, the EORTC has started a randomized study to test the contribution of chemotherapy to the achievement of local control.

Promising phase II studies in patients with malignant head and neck tumors have also been presented, suggesting that an increase in complete remissions can be achieved with 5-fluorouracil and cisplatin followed by radiotherapy (*15–18*). Most of these studies have not yet been confirmed in properly randomized phase III trials.

No improvement or a possible detrimental effect on survival after induction chemotherapy with cisplatin and 5-fluorouracil has been observed in two randomized phase III trials for patients with head and neck tumors (*19,20*). Both studies can be criticized on the grounds that they presented very preliminary results and that there was a large heterogeneity among the patient groups and treatments included. The results presented by both groups, however, should still be considered as a warning of the possible dangers of the use of combination radiotherapy and chemotherapy in these tumor sites outside clinical trials.

FAILURE OF TRIALS

The fact that combined treatment has not proved to be better than single modality treatment or has failed completely in a number of trials can be attributed to biologic or clinical factors, trial design, and lack of quality control in performance of trials. In experimental studies showing that certain drugs in vitro and in vivo enhanced tumor cell kill, there has not been thorough investigation with regard either to their effects in a range of human tumor cell lines or to the damage from fractionated irradiation in early- and late-responding normal tissues.

Often, radiosensitizing properties of certain drugs have been tested in only a few animal tumor cell lines. A large scatter in radiosensitizing properties of cisplatin, for instance, could be mainly attributed to the sensitivity of certain cell lines to cisplatin (*21*). In addition, the failure of cytostatic drugs to act as radiosensitizers can be explained by the limited concentration of drug in the tumor at the time of irradiation because of limited uptake or rapid disappearance of the active metabolite of the drug. For example, increasing doses of cisplatin were given once a week to patients with local inoperable rectal cancer to evaluate the radiosensitizing properties of cisplatin in this tumor type in a dose-finding study at The Netherlands Cancer Institute. Regular biopsies were taken from the tumors before, during, and after treatment. It could be shown that nearly no platinum, as measured with the pixe method (*22*), was available at the time of irradiation (Dewit L, personal communication). No consecutive phase II and phase III trials in this tumor site were therefore initiated by our group. Related to this drug concentration problem is the influence of the time sequence of radiotherapy and chemotherapy. The radiosensitizing properties of cisplatin disappeared when it was given >48 hours before fractionated irradiation (*23,24*). This suggests that most of the cisplatin-induced DNA lesions responsible for the interaction of radiotherapy-chemotherapy were repaired in 48 hours.

Until recently, the normal tissue damage after fractionated irradiation and chemotherapy had not been investi-

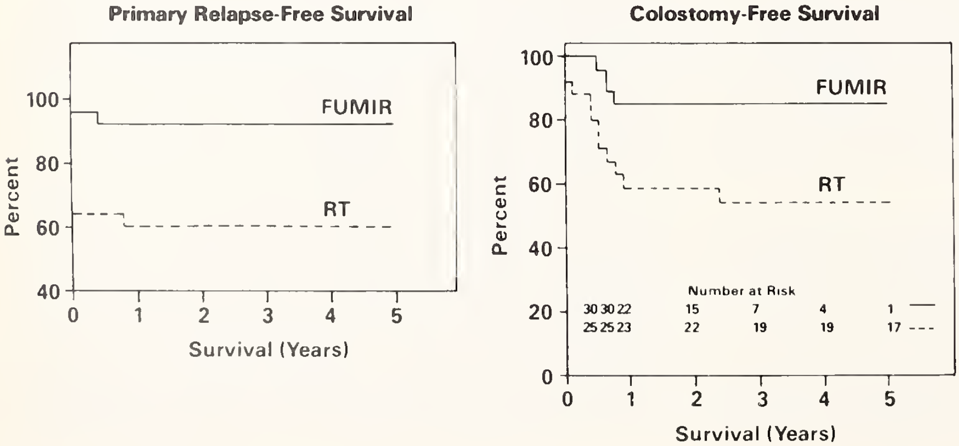


FIGURE 2.—Actuarial primary tumor control and colostomy-free survival from date of 1st irradiation for patients with anal carcinoma treated with high-dose radiation alone (RT) or combined with 5-fluorouracil and mitomycin (FUMIR). Reprinted with permission from (*13*).

gated very thoroughly. In early-responding normal tissues, drugs like cisplatin (*25,26*) and bleomycin (*27*) have been shown to increase the response to radiation, but this effect is only additive. The results described with bleomycin (*27*) are in agreement with the observations made in the study of oropharyngeal cancer conducted by the EORTC (*10*). In this trial, increased acute mucositis led to delay of the final radiation treatments. Dewit et al. (*28*) observed no significant increase in late rectal damage, but Stewart et al. (*29*) and Tanabe (personal communication) demonstrated a modest increase of late normal tissue damage in the kidneys and lungs, respectively, with DMF values ≤1.3 (table 1). The gain in local control in clinical trials with these drugs could therefore be limited by increased acute toxicity, resulting in a reduced radiation dose or late toxic effects.

The design of clinical trials and the limited number of patients have also considerably reduced the chance of detecting differences in these trials, particularly considering the large heterogeneity in the location of the primary tumor and the types of treatment used in the head and neck area. In addition, rigid quality control has not been performed in most of these trials. It can be expected that any possible gain may be lost by the large scatter of doses given, which is caused by differences in tissue density in the target area and by treatment planning and dosimetry, as demonstrated by Johansson et al. (*30*) in a quality control study.

IMPROVEMENT OF LOCAL CONTROL

Although no large contribution has been achieved by adding chemotherapy to conventional radiotherapy, improvement of the results can be expected by increasing the drug concentration in the tumor during irradiation, better selection of patients for the combined treatment by using predictive assays, and avoidance of the errors described here.

For combined treatment of the primary tumor, drug targeting is an important way to achieve higher tumor concentration of drugs. Monoclonal or polyclonal antibodies may be effective cytostatic drug carriers in the future. So far, however, increased drug uptake by no more than a factor of 2 has been achieved, which is probably insufficient for obtaining markedly improved results with radiosensitizers (*31*).

TABLE 1.—DMF for combination of cisplatin and fractionated irradiation

	End point	DMF[a]	Investigator (ref. No.)[b]
Tumor	Growth delay	1.9–2.2	Bartelink et al. (*25*) Tanabe[c]
Kidney	Late toxic effects	1.3	Stewart et al. (*29*)
Lung	Late toxic effects	1.3	Tanabe[c]
Small bowel	Early toxic effects	1.3	Bartelink et al. (*25*) Dewit et al. (*26*)
Large bowel	Late toxic effects	1	Dewit et al. (*28*)

[a] DMF are derived from irradiation schedules with ≥5 fractions and equal cisplatin doses.

[b] Studies were published in 1986.

[c] Personal communication.

A more direct way to increase the drug concentration in the tumor is intra-arterial infusion of the drug itself or of drug encapsulated in carriers such as microspheres, which are trapped in the vessels of the tumor. This approach will probably be the most effective in well-vascularized tumors (*32*).

Implantation of the drug into the tumor is another direct way to increase the drug concentration during treatment. For optimum results, these drugs should be incorporated in slow-release systems to maintain a high drug concentration over the long treatment time of fractionated radiotherapy. In an ideal system, very high tumor concentrations of the drugs can be obtained over long treatment periods, with relatively low plasma levels. This means that, compared with iv injections, much higher tumor concentrations of the sensitizers can be achieved with the same or decreased toxic effects (*33*). Intratumoral implantation of a drug incorporated in a slow-drug-release system has the added advantage that the drug is likely to reach tumor cells in which the blood supply and oxygen access are limited. This approach has the same advantages as implantation of radioactive sources; i.e., the treatment volume is limited and the surrounding normal tissues can be spared. Thus, the adjacent normal tissues can be spared with both methods.

These methods with potential for improvement of local control should be evaluated by the results of pharmacokinetic studies. Intracellular measurements of the drug in human tumor biopsies and, if possible, on the target (e.g., DNA) are especially necessary for prediction of whether a cytostatic drug can be successfully used as a radiosensitizer. An example of such an intracellular measurement is the recently demonstrated binding of cisplatin to its putative target DNA in cryostat tissue sections of rats treated with cisplatin, using an immunocytochemical technique (*34*). This assay could be useful to determine drug penetration in solid tumors and to correlate the formation and persistence of drug–DNA adducts to radiosensitizing effects.

A clinical possibility for benefit from the combination of chemotherapy and radiotherapy is the use of the chemotherapy response to predict the results of radiotherapy. Studies from Al-Sarraf et al. (*15*), Weaver et al. (*16*), and Ensley et al. (*17*) showed that in patients who achieved complete remission after chemotherapy, recurrences almost never occurred within the radiotherapy field. Most of the patients who did not have a response to chemotherapy developed a recurrence in the irradiated area. Based on this prognostic information, patients can be selected to receive radiotherapy, and mutilating surgery can be reserved for the nonresponders. These possibilities are now under study in a few trials; one such trial is EORTC study 24681, a randomized phase III trial in which patients with T3 laryngeal cancer will receive either laryngectomy or chemotherapy followed by radiotherapy. Disadvantages of such an approach are the toxicity of the chemotherapy and the delay of treatment in the nonresponders. The latter disadvantage may have a detrimental effect on the survival curve of the whole group (*16,17*).

Sensitive methods are badly needed to predict the success of radiotherapy and chemotherapy. The methods of investigation described by Brock et al. (*35*) are a promising approach to predict a successful outcome in individual patients. With this assay, either direct tumor biopsies can be taken from patients before and after treatment and cell survival can be rapidly assessed or the specimen can be irradiated in vitro with low radiation doses as an intrinsic radiosensitivity test.

SELECTION CRITERIA

Treatment Schedule

The rationale behind new treatment proposals should form the basis for designing treatment schedules. For the combination of radiotherapy and chemotherapy, the rationale can be 1) sensitization of the radiation effects; 2) additive cell killing; and 3) reduction of the number of tumor cells before irradiation. For example, if one selects combined treatment to enhance response to radiation, with a cytostatic drug acting as a radiosensitizer, the drug should be given shortly before each radiation treatment to achieve the highest tumor concentration available at the moment of irradiation. To avoid serious side effects such as mucositis or bone marrow toxicity, we may find it necessary to change the conventional 7-week treatment schedule to multiple fractions per day over short periods, with the addition of rest periods. This approach has been explored by the EORTC in a randomized phase II study in patients with malignant head and neck tumors.

If the rationale behind the study is based on reduction of the tumor volume with sparing of normal tissues by combining two modalities that have differences in toxicity to normal tissues, a good method may be alternation of radiotherapy and chemotherapy to allow the normal tissues to recover from the specific toxic effects of each treatment modality (*36–38*).

Patients

It can be argued that using cytostatic drugs as radiosensitizers will not be successful if only a very few patients are cured locally with radiation alone. In these radioresistant tumors, the maximum tolerated dose for radiation is within the early, shallow part of the radiation dose-effect curve (fig. 3). This means that a cytostatic drug or another sensitizer with a DMF $\leq$1.6 will have only a limited effect on improvement of local control. However, successful use of cytostatic drugs as radiosensitizers can be achieved in moderately sensitive tumors, i.e., 20%–50% local control with radiotherapy alone. This means that the local control rate is already in the steep part of the dose-effect curve. It can therefore be expected that with a DMF $>$1.3, considerably more cures can be achieved. The patient groups selected for combined treatment should therefore be those with tumor types that already have a reasonable chance of local cure. In addition, these tumor types should be chemosensitive, with a sufficient number of partial and complete responders, or these tumors should incorporate the drug in sufficient quantities to achieve drug levels that have been shown to cause enhancement of radiation effects in vitro. Because it is also necessary to select patients who can tolerate the aggressive approach of combined treatment, a good performance status is required.

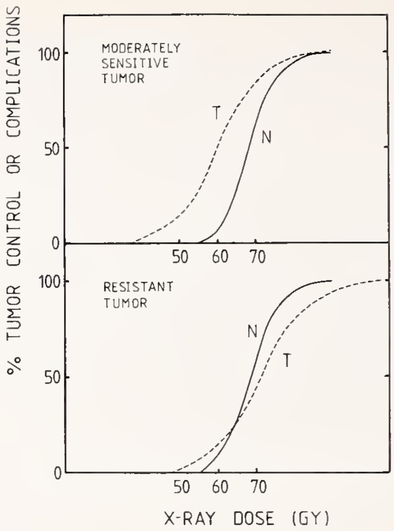


FIGURE 3.—DMF values $\leq$1.6 for radiosensitizers will have limited effect in radioresistant tumors because maximum tolerated dose in these patients is still in early, shallow part of dose-effect curve. T = tumor control; N = normal tissue complications.

CONCLUSIONS

Combined treatment with radiotherapy and chemotherapy has resulted in improved local control and sometimes survival in patients with breast cancer and anal cancer and in those with certain head and neck tumors. The better results were correlated with a reduction in the number of patients dying with uncontrolled local symptoms and the potential to avoid mutilating surgery. On the other hand, combined treatment failed to show any significant benefit in a number of trials, due to heterogeneity of patients and treatments and possibly to limited drug access to tumor cells.

For new approaches in combined modality treatment, it is therefore necessary that careful selection and stratification criteria should be used in clinical trials. The use of cytostatic drugs as radiosensitizers should be initiated only if the following requirements are fulfilled: sufficient intracellular drug uptake; sensitizing properties in human tumor cells; enhancement of tumor cell killing; and little or no increase in early and late radiation damage.

New techniques that predict the outcome for individual patients (e.g., response to chemotherapy) may be used to predict the results of radiotherapy. New approaches of drug targeting with monoclonal antibodies, intra-arterial infusion, and intratumoral implantation of slow-drug-release systems should result in much higher tumor drug concentration and less general exposure, i.e., decreased toxic effects for the patient.

REFERENCES

(*1*) SUIT HD, WESTGATE SJ: Impact of improved local control on survival. Int J Radiat Oncol Biol Phys 12:453–458, 1986.

(*2*) COX JD: Failure analysis in diagnostic and treatment strategies in cancer management. Cancer Treat Symp 2:1–4, 1983.

(*3*) BARTELINK H, BREUR K, HART G: Radiotherapy of lymph node metastases in patients with squamous cell carcinoma of the head and neck region. Int J Radiat Oncol Biol Phys 8:983–989, 1982.

(*4*) DEVITA VT, LIPPMAN M, HUBBARD SM, et al: The effect of combined modality therapy on local control and survival. Int J Radiat Oncol Biol Phys 12:487–501, 1986.

(*5*) Lo TC, Wiley AL, Ansfield FJ, et al: Combined radiation therapy and 5-fluorouracil for advanced squamous cell carcinoma of the oral cavity and oropharynx. A randomized study. Am J Roentgenol 126:229–235, 1976.

(*6*) Reagan TJ, Bisel HE, Childs DS, et al: Controlled study of CCNU and radiation therapy in malignant astrocytoma. J Neurosurg 44:186–190, 1976.

(*7*) Fu KK, Phillips TL, Silverberg IJ, et al: Combined radiotherapy and chemotherapy with bleomycin and methotrexate for advanced inoperable head and neck cancer: Update of a NCOG randomized trial. J Clin Oncol. In press.

(*8*) Shanta V, Krishnamurthi S: Combined bleomycin and radiotherapy in oral cancer. Clin Radiol 31:617–620, 1980.

(*9*) Vermund H, Kaalhuis O, Winther F, et al: Bleomycin and radiation therapy in squamous cell carcinoma of the upper aero-digestive tract: A phase III clinical trial. Int J Radiat Oncol Biol Phys 11:1877–1886, 1985.

(*10*) Cachin Y, Jortay A, Sancho H, et al: Preliminary results of a randomized EORTC study comparing radiotherapy and concomitant bleomycin to radiotherapy alone in epidermoid carcinomas of the oropharynx. Eur J Cancer 13:1389–1395, 1977.

(*11*) Carter SK: Adjuvant chemotherapy of cancer. A review of its current status. Drugs 31:337–367, 1986.

(*12*) Tannock IF, Browman G: Lack of evidence for a role of chemotherapy in the routine management of locally advanced head and neck cancer. J Clin Oncol 4:1121–1126, 1986.

(*13*) Cumming BJ, Keane TJ, Thomas GM, et al: Results and toxicity of the treatment of anal carcinoma by radiation therapy or radiation therapy and chemotherapy. Cancer 54:2062–2068, 1984.

(*14*) Papillon J: Current therapeutic concepts of management of carcinoma of the anal canal. Presented at the Fifth Annual Meeting of the European Society for Therapeutic Radiology and Oncology, Baden-Baden, Federal Republic of Germany, 1986.

(*15*) Al-Sarraf M, Drelichman A, Peppard S, et al: Adjuvant *cis*-platinum and 5-fluorouracil 96 hour infusion in previously untreated epidermoid cancers of the head and neck. Proc Am Assoc Cancer Res ASCO 22:428, 1981.

(*16*) Weaver A, Flemming S, Kish J, et al: *cis*-Platinum and 5-fluorouracil as induction therapy for advanced head and neck cancer. Am J Surg 144:445–448, 1982.

(*17*) Ensley J, Jacobs J, Weaver A, et al: Initial cisplatin combination predicting response to radiotherapy in patients with advanced epidermoid cancers of the head and neck. Proc ASCO 1:197, 1982.

(*18*) Fee WE Jr, Jacobs C, Goffinet DR, et al: Selection of favorable patients with unfavorable head and neck cancers. Presented at the Fifth Joint Meeting of the American Society for Head and Neck Surgery and the Society of Head and Neck Surgeons, Dorado Beach, Puerto Rico, May 1985.

(*19*) Martin M, Mazeron JJ, Glaubiger D, et al: Neoadjuvant polychemotherapy of head and neck cancer: Preliminary results of a randomized study. Proc ASCO 5:141, 1986.

(*20*) Haas C, Anderson T, Byhardt R, et al: Randomized neoadjuvant study of 5-fluorouracil (FU) and *cis*-platinum (DDP) for patients (PTS) with advanced resectable head and neck squamous cancer (ARHNSC). Proc Am Assoc Cancer Res 27:185, 1986.

(*21*) Begg AC, Van der Kolk PJ, Dewit L, et al: Radiosensitization by cisplatin of RIF1 tumour cells in vitro. Int J Radiat Biol 50:871–874, 1986.

(*22*) Dikhoff TGMH, Van der Heide JA, McVie JG: Topographic pixe analysis of platinum levels in kidney slices from cisplatin-treated patients. Nucl Instruments Methods Phys Res B10/11:639–642, 1985.

(*23*) Begg AC, Van der Kolk PJ, Bartelink H: Radiosensitization in vitro by the two cisplatin analogues diammine-(1,1-cyclobutanedicarboxylate)platinum (II) and ethylenediamminemalomatoplatinum (II). Radiother Oncol. In press.

(*24*) Lelieveld P, Scoles MA, Brown JM, et al: The effect of treatment in fractionated schedules with the combination of X-irradiation and six cytostatic drugs on the RIF-1 tumor and normal mouse skin. Int J Radiat Oncol Biol Phys 11:111–121, 1985.

(*25*) Bartelink H, Kallman RF, Rapachietta D, et al: Therapeutic enhancement in mice by clinically relevant dose and fractionation schedules of *cis*-diamminedichloroplatinum (II) and irradiation. Radiother Oncol 6:61–74, 1986.

(*26*) Dewit L, Oussoren Y, Bartelink H: Dose and time effects of *cis*-diamminedichloroplatinum (II) and radiation on mouse duodenal crypts. Radiother Oncol 4: 363–372, 1985.

(*27*) Vanuytsel L, Feng Y, Landuyt W, et al: The combined effect of bleomycin and irradiation on mouse lip mucosa. 2. Influence on the accumulation and repair of sublethal damage during fractionated irradiation. Radiother Oncol 6:267–273, 1986.

(*28*) Dewit L, Oussoren Y, Bartelink H: Early and late damage in the mouse rectum after irradiation and *cis*-diamminedichloroplatinum (II). Radiother Oncol 8:57–69, 1987.

(*29*) Stewart F, Bohlken S, Begg A, et al: Renal damage in mice after treatment with cisplatinum alone or in combination with X-irradiation. Int J Radiat Oncol Biol Phys 12:927–933, 1986.

(*30*) Johansson KA, Horiot JC, van Dam J, et al: Quality assurance control in the EORTC cooperative group of radiotherapy. 2. Dosimetric intercomparison. Radiother Oncol 7:269–279, 1986.

(*31*) Goldenberg DM, Kim EE, Deland FH, et al: Radioimmunodetection of cancer with radioactive antibodies to carcinoembryonic antigen. Cancer Res 40:2984–2992, 1980.

(*32*) Tomlinson E, Burger JJ, Schoonderwoerd EMA, et al: Human serum albumin microspheres for intraarterial drug targeting of cytostatic compounds. Pharmaceutical aspects and release characteristics. *In* Microspheres and Drug Therapy, Pharmaceutical, Immunological and Medical Aspects (Davis SS, Illum L, McVie JG, et al, eds). Amsterdam: Elsevier Science Publishers, 1984, pp 75–89.

(*33*) Begg AC, Bartelink H, Stewart FA, et al: Improvement of differential toxicity between tumor and normal tissues using intratumoral injection with or without a slow-drug-release matrix system. NCI Monographs 6:133–136, 1988.

(*34*) Terheggen PMAB, Floot BCJ, Scherer E, et al: Immunocytochemical detection of interaction products of *cis*-diamminedichloroplatinum(II) (*cis*-DDP) (*cis*-diammine-(1,1-cyclobutanedicarboxylato)platinum(II) (CBDCA) with DNA in rodent tissue sections. Cancer Res. In press.

(*35*) Brock WA, Maor MH, Peters LJ: Predictors of tumor response to radiotherapy. Radiat Res 104:S290–S296, 1985.

(*36*) Tubiana M, Arriagada R, Cosset JM: Sequencing of drugs and radiation. The integrated alternating regimen. Cancer 55:2131–2139, 1985.

(*37*) Looney WB, Hopkins HA, Carter WH: Solid tumor models for the assessment of different treatment modalities. XXIII. A new approach to the more effective utilization of radiotherapy alternated with chemotherapy. Int J Radiat Oncol Biol Phys 11:2105–2117, 1985.

(*38*) Hoppe RT, Portlock CS, Glatstein E, et al: Alternating chemotherapy and irradiation in the treatment of advanced Hodgkin's disease. Cancer 43:472–481, 1979.

An Organized Multi-institutional Interdisciplinary Evaluation of Role of Radiation Therapy Alone or Combined With Chemotherapy in Treatment of Adenocarcinoma of the Gastrointestinal Tract[1]

H. O. Douglass, Jr.,[2,*] D. M. Stablein,[3] and P. R. M. Thomas[4]
For the Gastrointestinal Tumor Study Group

ABSTRACT—Adenocarcinomas of the gastrointestinal tract have generally been considered to be radioresistant. In 1974-1975, following an early lead from the Mayo Clinic (Rochester, MN), the Gastrointestinal Tumor Study Group initiated a series of clinical trials of radiation therapy and chemotherapy as surgical adjuvant programs for patients with pancreatic and rectal cancer and for the treatment of locally unresectable gastric and pancreatic adenocarcinomas. The first protocols for pancreatic cancer included a controlled trial of radiation therapy and chemotherapy following pancreatoduodenectomy or total pancreatectomy and also a randomized trial of high-dose radiation therapy, with or without chemotherapy, compared to a lower dose of radiation therapy combined with chemotherapy for patients with locally unresectable tumors. In the treatment of locally incurable gastric cancer, radiation therapy plus chemotherapy was compared to chemotherapy alone, while the rectal trial was a randomized comparison of radiation therapy; chemotherapy; the combination of radiation therapy and chemotherapy; and no further treatment following surgical extirpation. In all cases, the agent used during the course of radiation was 5-fluorouracil. Subsequent trials in pancreatic cancer compared radiation combined with either 5-fluorouracil or doxorubicin and included a pilot study of hyperfractionated radiation therapy combined with 5-fluorouracil. Confirmatory trials were undertaken and are still under analysis in gastric cancer and in rectal cancer. A follow-up trial in pancreatic cancer was developed to establish the importance of the radiation therapy component of combined modality therapy in the treatment of patients with locally unresectable disease. A final study examined the potential for radiation therapy of the liver and systemic chemotherapy in the prevention of metastatic adenocarcinoma of the colon. The results of the adjuvant trials showed that combined modality therapy enhanced the survival of patients following resection of pancreatic and rectal cancers. When used alone in rectal cancer, radiation therapy was less effective than combined modality therapy. Combined modality therapy enhanced survival of patients with locally unresectable pancreatic and gastric cancers. Hyperfractionation and the use of doxorubicin in place of 5-fluorouracil did not further increase survival of pancreatic cancer patients. When combined with hepatic irradiation, 5-fluorouracil was excessively toxic. However, in the adjuvant studies, 5-fluorouracil combined with radiation therapy had been well tolerated. These studies confirmed the efficacy of 5-fluorouracil as an adjuvant to radiation therapy in the treatment of gastrointestinal carcinoma.—NCI Monogr 6:253-257, 1988.

In 1973, realizing the paucity of available clinical research and treatment protocols for gastrointestinal cancer, and particularly the absence of organized programs of surgical adjuvant therapy, the National Cancer Institute called together a group of consultants from a number of universities and cancer centers to develop proposals for multidisciplinary therapy. As a result of a series of these meetings, the GITSG was formed. Its first mission was to develop a surgical adjuvant trial for the management of patients with pancreatic cancer and, simultaneously, to develop a program for the treatment of patients with locally unresectable disease, a stage comprising, at the time of diagnosis, nearly 40% of all patients with pancreatic cancer. In 1974, these first trials were initiated, to be followed soon thereafter by treatment programs for gastric cancer (1974) and colorectal cancer (1975).

It is quite remarkable that, of the initial four adjuvant studies from the GITSG, the gastric, pancreatic, and rectal studies should all have provided positive results, while the colon study challenged so-called historical treatment results because of the remarkable survival of patients entered in its control group. The initial studies for locally incurable disease in gastric and pancreatic cancer were similarly noteworthy in terms of positive results. Follow-up studies in pancreatic cancer confirmed the initial trial, and subsequent studies in gastric, pancreatic, colon, and rectal cancer are currently under evaluation. Most remarkable has been the demonstration of survival benefit resulting from radiation therapy for the randomized patients with gastric, pancreatic, and rectal cancer; these adenocarcinomas had previously been considered quite radioresistant.

The combined modality studies added the administration of 5-FU on the first 3 days of radiation therapy. Because concepts of radiation therapy at this time suggested that higher doses of radiation could be safely administered by splitting the total dose into multiple courses, the protocols utilized a series of radiation courses of 20 or 25 Gy administered to a total dose of 40–60 Gy in the various disease sites. When the course of radiotherapy was split, 5-FU was administered on the first 3 days of each course. When radiation therapy was given in a single continuous course, 5-FU

ABBREVIATIONS: GITSG = Gastrointestinal Tumor Study Group; 5-FU = 5-fluorouracil.

---

[1] Supported in part by Public Health Service grant CA-34184 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Roswell Park Memorial Institute, Buffalo, NY.

[3] Emmes Corporation, Potomac, MD.

[4] Mallinckrodt Institute of Radiology, Washington University, St. Louis, MO.

[*] *Reprint requests:* Harold O. Douglass, Jr., M.D., Roswell Park Memorial Institute, 666 Elm St., Buffalo, NY 14263.

was administered on the first 3 and last 3 days of treatment. Generally, the 5-FU dose administered was 500 mg/m^2 of body surface area.

The administration of 5-FU was based on studies by Moertel et al. at the Mayo Clinic (*1*). In these studies, patients received either 5-FU or a placebo, along with radiation therapy of gastric, pancreatic, and rectal cancers. Patients who received 5-FU had a significantly longer survival than those who did not, a phenomenon that was noted in all three tumor sites.

PANCREATIC CANCER

The series of studies of the GITSG in locally unresectable pancreatic cancer showed that 5-FU combined with radiation therapy was more effective than radiation therapy alone, while in the adjuvant study, following potentially curative resection, combined modality therapy was more effective than no antineoplastic treatment (*2*). Increasing the intensity of radiation therapy through hyperfractionation or enhancing the radiopotentiation by the use of doxorubicin did not further increase survival of patients with locally unresectable disease.

In the initial trial for treatment of locally unresectable pancreatic cancer, a dose of 60 Gy was administered as a double split course with or without 5-FU (500 mg/m^2) administered iv on the first 3 days of each 20-Gy segment of radiation therapy (*3*). The third arm in this trial examined a dose of 40 Gy rather than 60 Gy administered as a single split course and combined with 5-FU (table 1). In both of the protocols in which 5-FU was administered during the course of the radiation therapy, weekly bolus infusion of 5-FU followed radiation for 2 years.

Radiation therapy alone in this trial had been discontinued after only 25 patients were entered in each arm because it became apparent that the median survival in this arm was approximately 50% of that in the other 2 arms, in which 5-FU was given along with radiation therapy. In the final analysis, only 14% of the patients who received radiation therapy alone lived 1 year, while 39%–44% of those who received radiation therapy combined with chemotherapy survived 1 year. The survival of patients in the combined modality arms was significantly longer than the survival of patients who received radiation therapy alone.

TABLE 1.—Radiation therapy alone or as part of combined modality treatment for locally unresectable pancreatic cancer[a]

Trial No.	Treatment program	No. of patients evaluated	Survival Median (wk)	Survival 1 yr	*P* value
73	60 Gy double split	25	23	14%	
	60 Gy double split + 5-FU	117	47	39%	0.001
	40 Gy split + 5-FU	111	40	44%	<0.01
77	60 Gy double split + 5-FU	73	37	26%	
	40 Gy continuous + Adr	70	33	33%	NS
99[b]	50.4 Gy hyperfractionated + 5-FU; then STZ, MMC, 5-FU	18			
83[b]	54 Gy + 5-FU; then STZ, MMC, 5-FU weekly	22			
	STZ, MMC, 5-FU weekly	21			

[a] Adr = doxorubicin; STZ = streptozocin; MMC = mitomycin; NS = not significant.

[b] Analysis incomplete (recent trial).

It was thought that doxorubicin might be a more effective potentiator of radiation therapy. The drug had been shown to be a minimally effective agent in the treatment of advanced pancreatic cancer, and a pilot study had suggested that radiation therapy combined with doxorubicin might be quite an effective therapy. As a result, 143 patients were randomized into a study in which they received either the "best" of the three treatments from the earlier study or a dose of 40 Gy administered in a continuous course, with doxorubicin given on the first day of treatment (15 mg/m^2) and then on each Monday in a low-dose regimen (10 mg/m^2). Following completion of radiation therapy, doxorubicin was given on a 3- to 4-week schedule until a "maximum safe" dose had been administered, at which time the chemotherapy was changed to 5-FU (*4*).

Unfortunately, doxorubicin did not enhance survival (table 1). While slightly more patients survived 1 and 2 years following treatment with doxorubicin plus radiation, the median survival was shorter than that in the patients treated with radiation and 5-FU. There was no significant difference between the two survival patterns, but toxicity was considerably greater with doxorubicin.

Another approach was to administer 50.4 Gy, giving the treatments twice daily combined with 5-FU on the first 3 and last 3 days of treatment and then following a 1-month rest interval with streptozocin, mitomycin, and 5-FU (*5*). This three-drug combination has been shown in advanced disease studies to provide a longer survival than has been seen in any of a series of 19 other treatments. Unfortunately, this pilot program, which contained only 18 patients, failed to enhance median survival (table 1). Only 20% of the patients survived 1 year.

Since it did not appear that survival was enhanced by increased radiation therapy, by increasing either the total dose, the intensity of radiation, or the "quality" of the radiopotentiating agent, it was logical to test the combination of radiation therapy and chemotherapy against chemotherapy alone. A previous study of the Eastern Cooperative Oncology Group had suggested that radiation therapy plus 5-FU was no better than 5-FU alone for the treatment of patients with pancreatic cancer (*6*). These results were to be challenged by the GITSG because the radiation therapy program of the Eastern Cooperative Oncology Group study, by 1983 standards, was less than optimal and because chemotherapy better than 5-FU alone could probably be administered. This study has not been completely analyzed, but preliminary review suggests a survival advantage for patients who received radiation therapy plus chemotherapy, compared to those who received chemotherapy alone.

One might ask whether there was any information to suggest that treatment was better than no treatment at all. The investigators of the GITSG, along with investigators in other groups, did not think it was ethical to leave patients with known pancreatic cancer masses untreated. Thus, the only trial that could show that treatment was better than no treatment would involve patients receiving adjuvant therapy, those in whom an apparent "curative" resection of a cancer had been performed.

Within 6 weeks following a pancreatic resection, patients were offered the opportunity to participate in a trial that randomized them to receive 5-FU and radiation therapy (40 Gy in a split course); this treatment was similar to that administered in the local disease trial that began in 1973. The alternative was supportive care only (*2*).

Surgeons were slow to enter their patients into this study, fearing that in the postoperative period, radiation therapy plus 5-FU in a program this aggressive would not be tolerated. An additional factor slowing accrual was the relative infrequency of pancreatoduodenal resection or total pancreatectomy for pancreatic cancer. As a result, only 43 evaluable patients were accrued over a period of 9 years. However, the survival patterns noted among those 43 patients were quite startling. One-half of the control subjects survived 1 year, but by 2 years, only 18% were alive, and at 5 years, only 1 patient survived (table 2). In contrast, of the patients who received radiation therapy plus chemotherapy, 67% were alive at 1 year, 43% at 2 years, and 18% at 5 years (*2*). Long-term follow-up showed that most of the patients who survived 5 years were still alive, whereas there are no survivors in the control group. The GITSG, realizing the frequency of reports of benefit in a small initial trial that could never be confirmed, instituted a second trial enrolling 30 consecutive patients to receive the treatment program of radiation therapy and chemotherapy. Much to the surprise of all, once a potential therapeutic benefit of adjuvant therapy in pancreatic cancer was demonstrated, a dramatic increase in the number of patients referred to the trial resulted in the 30 patients being accrued in little more than 2 years. While long-term follow-up is not yet available, preliminary data from this confirmatory group of patients suggest that the earlier prolonged survival seen in the randomized portion of the protocol is being confirmed.

TABLE 2.—Radiation therapy plus 5-FU as adjuvant treatment for pancreatic cancer

Treatment program	No. of patients evaluated	Survival 1 yr	2 yr	5 yr
Randomized trial				
40 Gy split + 5-FU on days 1–3; then 5-FU weekly	21	67%	43%	18%
Control	22	50%	18%	4%
Confirmatory trial[a]				
40 Gy split + 5-FU on days 1–3; then 5-FU weekly	30	77%		

[a] Analysis incomplete (recent trial).

GASTRIC CANCER

The suggestion from GITSG studies in gastric cancer is that radiation therapy plus chemotherapy, while potentially quite toxic, may provide very effective local control of residual disease.

In 1974, the GITSG initiated a trial in locally unresectable and unresected gastric cancer comparing 5-FU and semustine, the chemotherapy then thought to be the most effective in gastric cancer, to a program consisting of 50 Gy of radiation delivered in a split course, with 5-FU administered with each of the two 25-Gy segments. After a 4-week rest, chemotherapy with 5-FU and semustine was instituted; this treatment was the same as that administered in the group that received chemotherapy alone (*7*).

TABLE 3.—Radiation therapy plus chemotherapy vs. chemotherapy alone in treatment for locally unresectable or incompletely resected gastric cancer

Treatment program	No. of patients evaluated	Survival Median (wk)	1 yr	2 yr	5 yr
5-FU, semustine	45	70	70%	34%	6%
50 Gy + 5-FU split; then 5-FU, semustine	45	36	45%	24%	18%
5-FU, Adr, semustine[a]	45				
5-FU, Adr, semustine; then 43.2 Gy + 5-FU; then 5-FU, Adr, semustine[a]	46				

[a] Analysis incomplete (recent trial). Data still encoded.

Accrual to this trial was prematurely terminated when it became apparent that the median survival of the patients receiving chemotherapy alone was almost twice that of the patients receiving the combined modality therapy (table 3). On closer review, it became apparent that the patients who received combined modality therapy had much more severe weight loss and deterioration of performance status, as well as greater anorexia, nausea, vomiting, and hematologic toxicity. Thus, 10 of the 45 patients in the combined modality arm failed within the first 10 weeks of therapy, compared to only 3 in the group that received chemotherapy alone. The difference in survival seems to be almost entirely a matter of toxicity. At 2 years, the survival curves of the 2 arms differed by little more than the early toxicity and dropout in the combined modality arm. By 5 years, 18% of the patients who received combined modality treatment were still alive, compared to only 6% of those who received chemotherapy alone. It is important to remember that this trial was commenced at a time when reports following resection, presumably for cure of gastric cancer, noted little more than 20% long-term survival. With evidence of residual disease in each patient, this study strongly suggested a potential for long-term survival; 18% of the patients survived 5 years.

As a result of the long-term differences and crossing of the survival curves, a second trial was undertaken in an attempt to preserve the long-term survival while eliminating the early toxicity. Doxorubicin had been added to 5-FU and semustine because this three-drug combination had been found to be associated with superior survival in a series of GITSG trials in advanced disease. This new protocol randomized patients to receive the three-drug combination alone to a program of irradiation alternated with chemotherapy courses; patients received an initial full dose of chemotherapy followed at week 8 by 43.2-Gy continuous-course radiation therapy, with 5-FU administered on the first 3 and last 3 days of the radiation course. Subsequently, these patients received the three-drug chemotherapy combination, although at doses slightly reduced to avoid postradiation toxicity. Forty-five patients were entered into the chemotherapy arm, 46 into the combined modality arm. While the results of this trial are not yet available, preliminary evidence suggests that the median survival and the 1-year survival seen in the previous chemotherapy arm (5-FU and semustine) were probably replicated.

RECTAL CANCER

The rectal cancer trial of the GITSG provided perhaps the best evaluation of the potential of radiation therapy in a surgical adjuvant situation. In this study, patients received chemotherapy with 5-FU and semustine for 18 months; radiation therapy of 40–48 Gy in 4.5–5 weeks; or the combination of treatments consisting of 40–44 Gy plus 5-FU on the first 3 days of radiation therapy followed by 5-FU and semustine. These 3 treatment arms were randomized concurrently against the control arm, in which patients received no further antineoplastic therapy following surgical extirpation of the rectal neoplasm (*8*).

In this study of patients with Dukes B_2 and C rectal adenocarcinomas, radiation therapy plus chemotherapy reduced local recurrence, slightly reduced distant recurrence, and improved 5-year survival. The 5-year survival in the control group was 43%. Local recurrence was found in 24% of the patients in this group and distant recurrence in 34% (table 4).

Chemotherapy improved the 5-year survival to 56% of the patients by reducing distant recurrence but had no effect on local recurrence rates. Radiation therapy improved the 5-year survival to 52%, with a reduced incidence of local recurrence and, surprisingly, distant recurrence. However, the combination treatment increased the 5-year survival to 59%, reduced local recurrence to 11%, and reduced distant recurrence to 26%. Thus, combination therapy was superior to radiation therapy, to chemotherapy, and to the omission of adjuvant therapy in this study (*8*). The original published report suggested that only the disease-free survival of patients was prolonged, but follow-up data have demonstrated significant prolongation of overall survival (*9*).

Because of the leukemogenic potential of semustine, a second randomized study evaluated radiation therapy (identical between arms) plus 5-FU, followed either by 5-FU and semustine or by 5-FU alone. It is hoped that this study will define the role of semustine. If survival patterns of patients in the 2 treatment arms are identical, there becomes available for general use a treatment program that has been proven to reduce local recurrence and to prolong survival of treated patients with rectal adenocarcinoma. This follow-up study is particularly important since the combination of 5-FU and semustine has also been used in a number of studies in other cooperative groups. However, semustine is available only for active protocol studies, so the community physician is unable to apply the therapy developed in this study to patients.

TABLE 4.—Radiation therapy and chemotherapy in controlled trial comparing combined modality postoperative treatment to each component of combined modality therapy given alone

Treatment program	No. of patients evaluated	5-yr survival	Local recurrence	Distant recurrence
Control	58	43%	24%	34%
5-FU + semustine × 18 mo	48	56%	27%	27%
40–48 Gy in 4.5–5.5 wk	50	52%	20%	30%
40–44 Gy in 4.5–5 wk + 5-FU; then 5-FU + semustine to a total of 18 mo	46	59%	11%	26%

LIVER METASTASES

While the liver is a solitary site of metastatic disease in fewer than one-fourth of all patients with colorectal cancer, liver metastases constitute the major component of the morbidity and are usually the cause of mortality in these patients. The prevention of liver metastases or delay in postoperative occurrence would be of benefit in patients with colon cancer. Because the previous experience in the first study of adjuvant treatment of colon cancer by the GITSG suggested that the survival of patients with Dukes B_2 lesions (full-thickness penetration of the bowel wall without lymph node metastases) was nearly 80% of 5 years, patients with Dukes B_2 lesions were excluded from the second study. Only patients with lymph node metastases were offered treatment in this second adjuvant study, which was oriented toward the prevention of liver metastases.

In this protocol, patients were treated with curative resection followed by 21 Gy to the liver plus 5-FU or were randomized to a concurrent control group (*10*). An early result of this study was the remarkable and unexpected enhanced toxicity of 5-FU when administered to patients receiving hepatic irradiation. Two dose reductions were required before tolerable levels of 5-FU were found. The 5-FU is excreted through the biliary tree into an enterohepatic circulation. It is most likely that the excretion of the drug was inhibited by the radiation therapy, resulting in the enhanced toxicity.

Concepts of radiation therapy to the liver, particularly as an adjuvant therapy, are limited by the sensitivity of the normal hepatocyte to radiation. However, in an adjuvant situation, it was hoped that the lower dose of radiation would be effective on the small potential deposits of tumor cells that might be present, yet undetected, at the time of surgery or might implant in the liver as a result of their presence in the portal circulation after surgical manipulation. Careful analysis of this trial will be necessary. A major impact on survival cannot be expected because few patients die with metastatic disease confined to the liver. However, overall survival may be prolonged by the reduction in liver metastases or the delay in their growth, since in these patients, liver metastases are usually the determinant of survival. Studies of patterns of recurrence in the treated and control groups will be necessary once sufficient time has passed after closure of the study to allow a determination to be made.

DISCUSSION

The trials of the GITSG have suggested that combination of radiation therapy and chemotherapy is superior to radiation therapy alone, to chemotherapy alone, and to no active treatment in pancreatic and rectal cancer. The studies of Moertel et al. (*1*) appear to have been confirmed in that radiation therapy plus 5-FU was, in these studies, both well tolerated and more effective than radiation therapy alone for the treatment of gastrointestinal cancer. The results of the GITSG trials in pancreatic cancer suggest a definite approach to the management of the patient after pancreatoduodenectomy. In gastric cancer, they stress the need for a follow-up trial in which semustine is not involved, because of the potential for long-term survival of patients with localized disease treated by combined modality therapy. For patients with rectal cancer, the benefit of combined modality therapy as a practical treatment method will be determined by the results of the follow-up study, in which the unavailable carcinogenic drug semustine served as the only variable between the 2 treatment arms.

Conceptually, hyperfractionation has many advantages, but its use in the only known and admittedly small study in pancreatic cancer did not lend evidence to the theoretical potential for hyperfractionation in gastrointestinal neoplasia. It is apparent that 5-FU is as effective a radiation potentiator as any currently available. Perhaps further trials to enhance the effect of radiation therapy and chemotherapy should instead stress agents that might inhibit the repair of DNA during the weekend gaps of the standard radiotherapy course. An agent that is selective or semiselective in terms of effect on tumor cells compared to normal cells would be particularly valuable here.

The results of the GITSG studies suggest that radiation therapy should usually be used in the treatment of gastrointestinal neoplasia only as part of a multimodality regimen with 5-FU administered concurrently with the radiation therapy. An exception to this use of 5-FU would be radiation therapy in which the treatment fields involve significant proportions of the liver.

REFERENCES

(*1*) Moertel CG, Childs DS Jr, Reitemeier RJ, et al: Combined 5-fluorouracil and supervoltage radiation therapy of locally unresectable gastrointestinal cancer. Lancet 2: 865–867, 1969.

(*2*) Kalser MH, Ellenberg SS (Gastrointestinal Tumor Study Group): Pancreatic cancer: Adjuvant combined radiation and chemotherapy following curative resection. Arch Surg 190:899–903, 1985.

(*3*) Gastrointestinal Tumor Study Group: Therapy of locally unresectable pancreatic carcinoma: A randomized comparison of high dose (6000 rad) radiation alone, moderate dose radiation (4000 rad + 5-fluorouracil), and high dose radiation + 5-fluorouracil. Cancer 48:1705–1710, 1981.

(*4*) Gastrointestinal Tumor Study Group: Radiation therapy combined with Adriamycin or 5-fluorouracil for the treatment of locally unresectable pancreatic carcinoma. Cancer 56:2563–2568, 1985.

(*5*) Leichman L, Seydel HG, Stablein D: Continuous hyperfractionated radiation + 5-fluorouracil followed by streptozotocin, mitomycin-C and 5-FU for patients with localized unresectable cancer of the pancreas. Proc Am Assoc Cancer Res 25:774, 1984.

(*6*) Klaassen DJ, MacIntyre JM, Catton GE, et al: Treatment of locally unresectable cancer of the stomach and pancreas: A randomized comparison of 5-fluorouracil alone with radiation plus concurrent and maintenance 5-fluorouracil—An Eastern Cooperative Oncology Group study. J Clin Oncol 3:373–378, 1985.

(*7*) Gastrointestinal Tumor Study Group: A comparison of combination chemotherapy and combined modality therapy for locally advanced gastric carcinoma. Cancer 49:1771–1777, 1982.

(*8*) Gastrointestinal Tumor Study Group: Prolongation of disease-free interval in surgically treated rectal carcinoma. N Engl J Med 312:1465–1472, 1985.

(*9*) Douglass HO Jr, Mayer RJ, Lindblad AS, et al: Survival after postoperative combination treatment of rectal cancer. N Engl J Med 315:1294–1295, 1986.

(*10*) Kaplan RS, Steinberg SM: Enhanced myelotoxicity of combined 5-FU and hepatic radiation. Proc ASCO 3:557, 1984.

Phase III Trial of Irradiation Plus Chemotherapy for Patients With Hepatic Metastases and Hepatoma: Experience of the Northern California Oncology Group [1]

Michael A. Friedman,[2] Theodore L. Phillips,[3,*] John F. Hannigan, Jr.,[4] and Stephen K. Carter[5]

ABSTRACT—The effects of iv or intra-arterial chemotherapy added to hepatic irradiation were evaluated in a 3-arm randomized trial. Patients with predominantly hepatic metastases or with hepatoma were eligible. They were randomized to receive 2,100 cGy in seven fractions alone or with 5-fluorouracil given either intra-arterially or by iv infusion; doxorubicin and mitomycin were given by bolus simultaneously with the radiation in a single course. A total of 166 patients were entered in the study. Toxicity was acceptable, with no sign of enhanced radiation damage. Response was evaluated 4–6 weeks after treatment. No complete responses were seen, but partial responses ≥50% were observed in the groups treated with radiation only (17%), radiation plus drug given iv (25%), and radiation plus drug given intra-arterially (20%) ($P>.3$). Disease progression occurred in a larger number of patients who received radiation only (29%) at 6 weeks than in the other 2 groups (7% and 18%, respectively; $P<.03$). Thus, in terms of local response duration, the addition of chemotherapy enhanced the effect of the radiation. Survival was not different among the 3 groups.—NCI Monogr 6:259–264, 1988.

The refractoriness to conventional systemic chemotherapy in primary liver cancer or colon cancer disseminated to hepatic sites is well recognized, since <25% of patients benefit from such iv therapy (*1,2*). At the same time, preliminary attempts at focused multimodality therapy of hepatic metastases have been reported with somewhat more optimism. Various techniques, including external whole liver RT, IA chemotherapy, and combined modality approaches, have been reported to yield more satisfactory results than those achieved using iv chemotherapy. Previously, the NCOG explored a series of phase I and II studies utilizing IA chemotherapy and RT. In a stepwise progression, the dosage of RT was escalated to the tolerable level of 300 cGy/day (total dose, 2,100 cGy). The IA chemotherapy consisted principally of 5-FU (10 mg/kg per day × 7–9 days) and doxorubicin (3 mg/m^2 per day × 7 days) administered simultaneously with the RT. The details of these studies have been previously reported (*3–6*) but may be summarized as follows. There was objective partial response in 17 (43%) of the 40 patients with colorectal hepatic metastases who were treated and in 10 (42%) of the 24 patients with hepatocellular cancer. Relief of symptomatic hepatic pain was achieved in 48 (75%) of the 64 patients. Moderate, tolerable, and reversible toxic effects were encountered, and it was postulated that this combined modality therapy might be acceptably effective.

However, the need for a comparative phase III trial was apparent, to help determine the relative merits of the elements of this combined modality program. A 3-arm, prospectively randomized study was designed to evaluate the enhancement of hepatic RT by chemotherapy and to determine whether concomitant IA chemotherapy was superior to iv chemotherapy, both administered with RT. By keeping the RT and chemotherapy doses and schedules exactly the same in comparable arms, these clinical questions were addressed. The primary end point was tumor response in the liver.

METHODS

Over a period of 37 months, 166 patients were stratified and randomized in the phase III study. To be eligible for study, patients must have fulfilled the following criteria:

1) colorectal or hepatocellular adenocarcinoma or other primary carcinoma documented with biopsy confirmation;
2) surgical incurability and measurable liver metastases, with the liver being the major site of morbidity or life-threatening conditions;
3) Karnofsky performance status ≥50%;
4) no prior IA chemotherapy or hepatic irradiation;
5) adequate hematologic function defined as WBC count ≥4,000/mm^3 and platelet count ≥100,000/mm^3;
6) adequate hepatic function defined as bilirubin <7 mg/dl and prothrombin time <15 seconds;
7) adequate renal function defined as BUN <25 mg/dl and normal bilateral collecting systems, documented by iv pyelogram or CT scan;
8) no intrinsic cardiovascular disease, ascites, portal vein obstruction, or variceal bleeding; and
9) signed informed consent.

ABBREVIATIONS: RT = radiation therapy; IA = intra-arterial(ly); NCOG = Northern California Oncology Group; CT = computed tomography; 5-FU = 5-fluorouracil; FUDR = floxuridine.

---

[1] Supported by Public Health Service grant CA-21744 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and by an American Cancer Society Faculty Research Award.

[2] Clinical Investigations Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[3] Department of Radiation Oncology, University of California, San Francisco.

[4] Northern California Oncology Group, Belmont.

[5] Bristol-Myers Corp., New York, NY.

* *Reprint requests:* Theodore L. Phillips, M.D., CED 200, Department of Radiation Oncology, University of California, San Francisco, San Francisco, CA 94143.

Patients were stratified according to Karnofsky performance status, prior chemotherapy, and bilirubin level. They were randomly assigned to the treatment by telephone contact with the NCOG operations and statistical office. An adaptive randomization method of Efron (*7,8*) was used to achieve balance across stratification variables and institutions. The principal end point of the study was the response rate in the liver at 4–6 weeks as well as time to tumor progression in the liver. Overall survival was not of primary interest. Secondary therapies were given at the time disease progression was documented in the liver.

The initial staging workup included:

1) complete physical examination including measurement of liver size in three vertical planes;
2) chest radiograph;
3) complete blood cell count and chemical screening battery (SMA-12), prothrombin time, and ECG; and
4) CT scan of the abdomen or radionuclide liver scan to provide objective parameters of disease measurement and to assist in assessment of the quantity of disease.

Radiation Therapy

The target volume was the entirety of the liver. The treatment field extended from 1 cm above the dome of the right diaphragm to the right flank skin; 2 cm beyond the left lobe of the liver as defined by CT and simulation; and 2 cm below the inferior hepatic edge as defined by palpation, scan, and simulation. Port film verification was obtained at the initiation of therapy. When left lobe hepatomegaly extended substantially into the left upper quadrant, oblique fields or blocking of the lower two-thirds of the left kidney was employed to diminish the dose of radiation delivered to normal tissues such as the left kidney and the gastrointestinal tract. The daily fraction size was 300 cGy, and treatment was given in seven fractions to a total reference dose of 2,100 cGy over 7–9 days. Treatment was given on megavoltage machines, and all treatment details and port films were reviewed by T. L. Phillips. Radiation therapy and chemotherapy were begun simultaneously.

Intra-arterial Infusion Technique

For patients randomized to receive IA therapy, catheters were inserted into the femoral artery in the Department of Radiology and placed subselectively into the proper or common hepatic artery. In patients who had an unusual or dual blood supply, only the artery predominantly supplying the bulk of the tumor was cannulated. No attempt was made to embolize blood vessels or to use two simultaneous catheters in the same patient. Patients were confined to bed for the duration of this therapy, which was $\leq$9 days. Verification of catheter placement was made every other day prior to radiation therapy and on the last day of treatment or when doubt as to its location arose. This verification was done by diagnostic radiology or on the RT simulator, with or without contrast material. Using a bedside pump, the catheters were infused with standard dextrose or saline solutions with 4,000 units of heparin/liter.

Chemotherapy

Chemotherapy was begun simultaneously with RT for patients receiving either iv or IA therapy. The 5-FU was infused at a dosage of 10 mg/kg per day continuously for the entire time needed to give seven radiation treatments, usually 9 days. Doxorubicin was administered at 3 mg/m^2 per day for each day of RT (total dose, 21 mg/m^2). This dose was given to patients with bilirubin $\leq$1.5 mg/dl (2 mg/m^2 for 1.5–3 mg/dl and 1 mg/m^2 for $>$3 mg/dl). Mitomycin was given only on the first day of therapy at 8 mg/m^2 and was infused over 30 minutes IA or 3 minutes iv. If the WBC count fell to $<$2,500/mm^3 or if the platelet count was $<$75,000/mm^3, all therapy was temporarily discontinued. The catheter was left in place and therapy was reinstituted when the WBC count was $>$3,000/mm^3 and the platelet count was $>$100,000/mm^3.

Measurement of Effects

After the completion of therapy, patients were seen at 2-week intervals to evaluate hematologic and other toxicity. At 4–6 weeks after therapy, a repeat CT or nuclear scan and liver measurements were performed and options for further therapy were considered.

Radionuclide scan or CT scan of the liver as well as liver size were evaluated to determine response. Partial response was defined as $\geq$50% decrease in the size of hepatic lesions measurable on scan, lasting for $\geq$4 weeks (NCOG grade 3 response). The size of a lesion was determined by the product of the longest perpendicular diameters. If hepatomegaly was the primary indicator of disease, partial response was defined as a reduction in the sum of the liver measurements below each costal margin at the midclavicular lines and xyphoid by $\geq$30%. Minor response (NCOG grade 4) was defined as response less than grade 3, with tumor size clearly smaller than at the start of treatment (20%–49% regression). Stable disease (NCOG grade 5) was defined as tumor that does not meet the criteria for partial response but does not show evidence of disease progression. Progressive disease (NCOG grade 6) was defined as an increase $>$20% in the size of any measurable lesion, increased liver size, or persistent worsening of liver function abnormalities.

In addition to liver scan and liver size, changes in performance status, liver function, and weight were evaluated. Survival was measured from the randomization date until death of a patient. Time to disease progression was measured from the start of treatment until progression of liver disease.

Statistical Considerations

Comparisons of response rates were made by Fisher's exact test. Curves of survival and estimated time to disease progression were constructed according to the method of Kaplan and Meier and compared using the log-rank test (*9*).

Clinical and Evaluability Characteristics

A total of 166 patients were entered in the trial; 147 were evaluable for toxicity and response. One institution participated only in the RT and iv arms, which resulted in a greater number of patients in these groups. Clinical characteristics of the patients with colon and liver primary tumors at entry to the study are summarized in table 1. Characteristics and toxic effects for patients with primary tumors other than colon cancer and hepatoma are omitted for brevity but were similar.

TABLE 1.—Patient characteristics[a]

Characteristic	RT	RT+iv	RT+IA
Colorectal cancer			
No. of patients	32	30	21
Age (yr)			
Mean	57	59	64
Range	37–68	39–75	52–77
Sex			
Male	18	15	12
Female	14	15	9
Prior chemotherapy			
Yes	10	13	9
No	22	17	12
Bilirubin			
≤2 mg/dl	28	29	20
>2 mg/dl	4	1	1
Hepatocellular cancer			
No. of patients	13	12	12
Age (yr)			
Mean	56	61	56
Range	33–70	35–74	31–74
Sex			
Male	11	7	7
Female	2	5	5
Prior chemotherapy			
Yes	3	0	2
No	10	12	10
Biliburin			
≤2 mg/dl	9	10	11
≥2 mg/dl	4	2	1

[a] Unless otherwise specified, values=No. of patients. IA and iv indicate routes of chemotherapy.

TABLE 2.—Liver response to treatment[a]

	RT	RT+iv	RT+IA
Colorectal cancer			
Total	32	30	21
Evaluable	31	26	17
Partial response	5	3	
Minor response	7	9	7
Stable disease	12	12	6
Continuous disease progression	7	0	1
Hepatoma			
Total	13	12	12
Evaluable	11	11	11
Partial response	2	4	4
Minor response	3	4	1
Stable disease	2	1	4
Disease progression	4	2	2
Other primary sites			
Total	14	18	14
Evaluable	10	18	12
Partial response	2	5	1
Minor response	2	3	4
Stable disease	2	8	3
Disease progression	4	2	4
All sites combined			
Partial response	17%	25%	20%[b]
Partial+minor response	40%	55%	50%[b]
Treatment failure	29%	7%	18%[c]

[a] Unless otherwise specified, values=No. of patients.

[b] $P>.3$.

[c] For RT vs. RT+iv, $P<.03$; for RT vs. RT+IA, $P=.3$; for RT+iv vs. RT+IA, $P=.23$.

RESULTS

The clinical benefit of therapy was judged at an arbitrary time (approximately 4–6 wk) after completion of therapy, and these results in terms of response in the liver are summarized in table 2. In neither group were complete objective responses observed.

The number of partial responses in the chemotherapy groups was slightly greater than the number in the groups receiving RT only. These differences were not statistically significant. There were more cases of disease progression in the group receiving RT only, and when the results of all 3 primary site groups are pooled, the difference between disease progression in the group receiving RT or RT plus iv chemotherapy is statistically significant ($P<.03$).

Time to disease progression was plotted for each subset of patients by primary site and for the whole group of evaluable patients (fig. 1). The results favor the iv chemotherapy group in each case, largely due to early disease progression in the group receiving RT only. The curves are not statistically different ($P>.3$). Median time to treatment failure showed a similar trend, but this was not statistically significant (table 3).

Survival curves were calculated for each arm and for the total group (fig. 2). There was no statistically significant difference in the median survival or between the entire curves for the total group or for each site of the primary tumor. The median survival times were 22 weeks for RT alone, 26 weeks for RT plus iv chemotherapy, and 17 weeks for IA chemotherapy with RT. One-year survival rates were 23%, 15%, and 17%, respectively. These survival data are of limited value because the major end point of the study was local response.

In this study, 1 patient had a fatal treatment-related toxic effect. This was a significant complication of IA therapy in

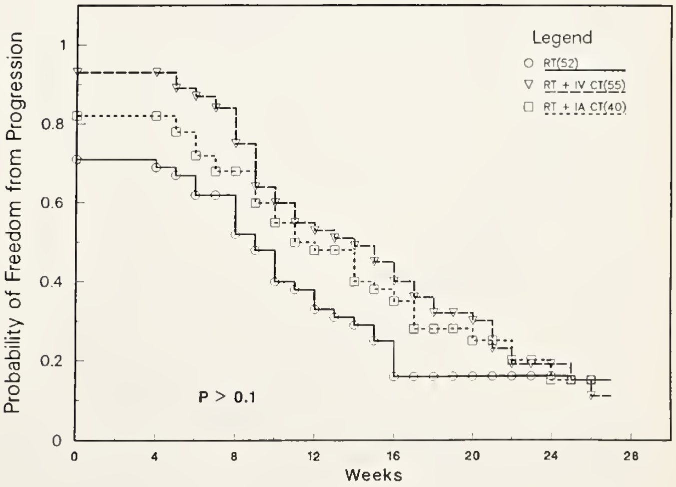


FIGURE 1.—Time to disease progression in all evaluable patients in study NCOG 3L91.

TABLE 3.—Median time to treatment failure in the liver

	RT		RT+iv		RT+IA	
	No. of patients	No. of wk	No. of patients	No. of wk	No. of patients	No. of wk
All cases	52	8.5	55	14	40	11
Hepatoma	11	4.5	11	10	11	7
Colon cancer	31	9.5	26	16	17	16
Other	10	8	18	10	12	6

TABLE 4.—Most severe hematologic and gastrointestinal toxicity[a]

Toxic effect	RT	RT+iv	RT+IA
Colorectal cancer			
Leukocytopenia ($\times 10^3/mm^3$)			
>4.5	16	5	3
3.0–4.4	10	16	7
2.0–2.9	4	5	6
1.0–1.9	1	0	1
<1.0	0	0	0
>130	15	15	9
Thrombocytopenia ($\times 10^3/mm^3$)			
90–129	8	4	0
59–89	5	4	5
25–49	2	2	1
<25	1	1	2
None	10	6	4
Nausea and vomiting			
Mild	8	10	5
Moderate	11	9	7
Severe	2	1	1
Hepatoma			
Leukocytopenia ($\times 10^3/mm^3$)			
>4.5	6	2	2
3.0–4.4	5	6	3
2.0–2.9	0	3	4
1.0–1.9	0	0	2
<1.0	0	0	0
>130	7	6	4
Thrombocytopenia ($\times 10^3/mm^3$)			
90–129	3	1	3
59–89	1	3	4
25–49	0	0	0
<25	0	1	0
None	3	2	3
Nausea and vomiting			
Mild	3	4	2
Moderate	4	4	4
Severe	1	1	2

[a] Values = No. of patients.

a patient who developed a clot in the catheter and died 1 week after its removal of thrombosis of the aorta and renal failure. Hematologic and gastrointestinal toxicity are displayed in table 4. No patient had leukopenia $\leq$1,000 cells/mm^3, but 5 patients had thrombocytopenia <25,000/mm^3. Nausea and vomiting were common and evenly distributed but severe in only 5 cases. No clinical radiation hepatitis or nephritis was encountered; it was not observed in 3 autopsied cases. Several patients in each treatment group had symptoms of peptic ulcer disease; 3 had gastrointestinal bleeding.

DISCUSSION

As a single modality, RT for hepatic metastases from colorectal cancer has been reported to be both palliative and capable of resulting in tumor regression. In studies performed >30 years ago, Phillips and co-workers (*10*) demonstrated that symptomatic palliation occurred in 26 (72%) of 36 patients receiving 2,000–3,750 cGy. Turek-Maischeider and Kazem (*11*) likewise demonstrated some efficacy. In 1978, Sherman and co-workers (*12*) described 55 patients who received 2,100–2,400 cGy; 24 (44%) had an "excellent response" of hepatic tumors. Several investigators have demonstrated that the liver will tolerate radiation doses <3,000 cGy without significant permanent hepatic injury. Despite these data, no controlled phase III trials had been performed, so the efficacy of this approach was not definitely known.

Radiation therapy is certainly not a novel modality for patients with hepatocellular cancer. Initial studies by Cohen and co-workers (*13*), and Phillips and Murikami (*14*) in the 1950s indicated the feasibility of this approach. Preliminary data from El-Domeiri et al. (*15*) demonstrated the safety of hepatic radiation doses of 1,000–3,600 cGy. Later data from Falkson (*1*), however, indicated no benefit for hepatic RT alone in hepatoma patients. In general, doses of radiation <2,000 cGy seemed ineffective, while doses >3,500 cGy were associated with a progressively higher risk of radiation hepatitis (*13–15*).

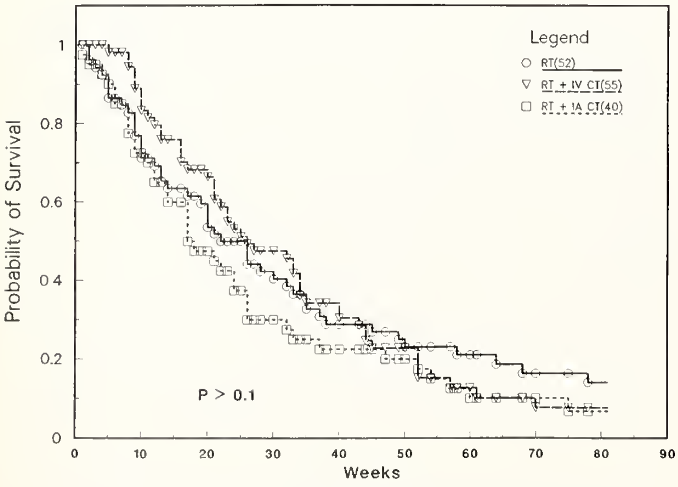


FIGURE 2.—Survival for all patients in study NCOG 3L91.

In a parallel manner, regional IA perfusion of fluoropyrimidines has become a recognized modality for hepatic metastases over the past two decades. Ansfield and co-workers (*16,17*) and Sullivan and Zurek (*18*) have demonstrated that 35%–85% of patients have some demonstrable tumor shrinkage with 5-FU or FUDR given IA. Nevertheless, the value of regionally infused chemotherapy for hepatic patients has been ill-defined. An extensive literature review has been provided by Haskell (*19*). Although the data are fragmentary, uncontrolled studies of IA fluoropyrimidines have reported efficacy for both hepatoma and colorectal cancer patients, with responses noted in 20%–40% (*2,19*). Pharmacologically, FUDR is thought to have a higher hepatic extraction ratio than 5-FU, perhaps contributing to this observed clinical effect (*20*). The use of other single agents through the hepatic artery and combination chemotherapy has been summarized by Friedman (*2*), and response rates of 40%–60% have been noted. Median survival of the overall group of patients who received IA chemotherapy reported in multiple series ranged from 150 to 600 days. It is not at all clear that any combination chemotherapy program is superior to a single agent used optimally. However, in the only prospective phase III study of 5-FU given IA versus iv, Grage et al. (*21*) were unable to demonstrate the superiority of IA over iv therapy. Criticism of this study has usually focused on the brevity (14 days) of the IA therapy and assertions that a longer infusion time would have been more efficacious.

More recently, combinations of chemotherapy and hepatic RT have been explored. In addition to the NCOG phase II data of 5-FU given IA plus doxorubicin, with 2,100 cGy, Webber et al. (*22*) reported that 8 (44%) of 18 patients responded to FUDR and radiation. Barone et al. (*23*) used an implantable device for prolonged IA administration of 5-FU or FUDR with 3,000 cGy; they demonstrated a median duration of survival of about 250 days in 18 patients. Liver function decompensation was noted in 6 patients and fatal radiation hepatitis in 1 patient. Obviously, there may be important pharmacologic, toxicological, and therapeutic implications of prolonged, indefinite 5-FU or FUDR therapy, which were not addressed in this current study.

Of interest are the data of Cochrane et al. (*24*), who studied hepatoma patients receiving either RT with subsequent chemotherapy or chemotherapy only. The chemotherapy consisted of 5-FU, cyclophosphamide, methotrexate, and vincristine. The radiation was given in 15 fractions of 200 cGy (total dose, 3,000 cGy over 3 wk). Median survival time was superior for the group receiving chemotherapy only (21 vs. 12 wk).

The current study was designed to address a limited question of enhancement of response to RT of the liver. It could be argued that different agents or schedules might have yielded superior responses (*20*). Moreover, anecdotal data suggest that long-term fluoropyrimidine perfusions may have substantially greater efficacy (*23,25*).

Recently, Leibel et al. (*26*) have reported on 214 patients treated with 2,100 cGy in seven fractions with or without misonidazole, which gave no benefit. Pain was relieved in 80% of the patients. The CT-based partial response rate was 7%, the minor response rate was 13%, and the median survival was 18 weeks. In the present study, the results in the chemotherapy arms compare favorably with those of Leibel et al., suggesting that combined modality therapy is to be preferred.

Given these considerations, we can conclude the following from this study. First, RT alone is not greatly different from RT plus chemotherapy in response induction, but it does result in more early treatment failures (rapid disease progression). Second, in patients receiving RT, IA chemotherapy is not superior to iv therapy for short-course treatments. Third, none of these therapies offers a substantial clinical antitumor effect. Obviously, considerable further investigation is required to fully define the ideal combination of focused (regional) RT and chemotherapy.

REFERENCES

(*1*) Falkson C: The treatment of liver cell cancer. *In* Liver Cell Cancer (Cameron DM, Linsell DA, Warwick GP, eds). Amsterdam: Elsevier, 1976, pp 81–93.

(*2*) Friedman MA: Primary hepatocellular cancer—Present results and future prospects. Int J Radiat Oncol Biol Phys 9:1841–1850, 1983.

(*3*) Friedman MA, Volberding PA, Cassidy MJ, et al: Therapy for hepatocellular cancer with intrahepatic arterial Adriamycin and 5-fluorouracil combined with whole-liver irradiation: A Northern California Oncology Group study. Cancer Treat Rep 63:1885–1888, 1979.

(*4*) Volberding PA, Friedman MA, Phillips TL: Hepatoma treated with intra-arterial (IA) polychemotherapy plus whole liver radiation. Proc Am Assoc Cancer Res ASCO 21:418, 1980.

(*5*) Friedman MA, Cassidy M, Levin M, et al: Combined modality therapy of hepatic metastasis. Cancer 44:906–913, 1979.

(*6*) Volberding PA, Friedman MA, Phillips TL, et al: Therapy of liver tumors metastatic for colorectal cancer with whole liver radiation combined with 5-FU, Adriamycin, and methotrexate. Cancer Chemother Pharmacol 9:17–21, 1982.

(*7*) Efron B: Forcing a sequential experiment to be balanced. Biometrika 58:403–417, 1971.

(*8*) Efron B: Randomizing and balancing a complicated sequential experiment. *In* Biostatistics Casebook (Miller R, Brown E, Efron B, et al, eds). New York: Wiley, 1980, pp 19–30.

(*9*) Peto R, Peto J: Asymptotically efficient rank invariant test procedures. J R Stat Soc 135:185–198, 1972.

(*10*) Phillips R, Karnofsky DA, Hamilton LD, et al: Roentgen therapy of hepatic metastases. Am J Roentgenol Radium Ther Nucl Med 71:826–834, 1954.

(*11*) Turek-Maischeider M, Kazem I: Palliative irradiation for liver metastases. JAMA 232:625–628, 1975.

(*12*) Sherman DM, Weichselbaum R, Order SE, et al: Palliation of hepatic metastasis. Cancer 41:2013–2017, 1978.

(*13*) Cohen L, Shapiro MP, Keen P, et al: Experimental radiotherapy of abdominal cancer: Radiosensitivity of liver tumors. Br J Radiol 27:402–406, 1954.

(*14*) Phillips R, Murikami K: Primary neoplasms of the liver: Results of radiation therapy. Cancer 13:714–720, 1960.

(*15*) El-Domeiri AA, Huros AG, Goldsmith HS, et al: Primary malignant tumors of the liver. Cancer 27:7–11, 1971.

(*16*) Ansfield FJ, Ramirez G, Davis HL, et al: Further clinical studies with intrahepatic artery infusion with 5-fluorouracil. Cancer 36:2413–2417, 1975.

(*17*) Ansfield FJ, Ramirez G, Skibba JL, et al: Intrahepatic arterial infusion with 5-fluorouracil. Cancer 28:1147–1151, 1971.

(*18*) Sullivan RD, Zurek WA: Chemotherapy for liver cancer by protracted ambulatory infusion. JAMA 194:481–486, 1965.

(*19*) Haskell CM: Cancer of the liver. *In* Cancer Treatment (Haskell CM, ed). Philadelphia: Saunders, 1980, pp 319–357.

(*20*) Ensminger WD, Rosowsky A, Raso V, et al: A clinical-pharmacological evaluation of hepatic arterial infusion of 5-fluoro-2′-deoxyuridine and 5-fluorouracil. Cancer Res 38:3784–3792, 1978.

(*21*) Grage TB, Vassilopoulis PP, Shingleton WW, et al: Results of a prospective randomized study of hepatic infusion with 5-fluorouracil versus intravenous 5-fluorouracil in patients with hepatic metastases for colorectal cancer: A Central Oncology Group study. Surgery 86:550–555, 1979.

(*22*) Webber BM, Soderberg CH Jr, Leone LA, et al: A combined treatment approach to management of hepatic metastases. Cancer 42:1087–1095, 1978.

(*23*) Barone RM, Byfield JE, Goldfarb PB, et al: Intraarterial chemotherapy using an implantable infusion pump and liver irradiation for the treatment of hepatic metastases. Cancer 50:850–862, 1982.

(*24*) Cochrane AMG, Murray-Lyon IM, Brinkley DM, et al: Quadruple chemotherapy versus radiotherapy in treatment of primary hepatocellular carcinoma. Cancer 40:609–614, 1977.

(*25*) Ensminger W: Effective control of liver metastases from colon cancer with an implanted system for hepatic arterial chemotherapy. Proc ASCO 1:94, 1982.

(*26*) Leibel SA, Pajak TF, Massullo V, et al: A comparison of misonidazole sensitized radiation therapy to radiation therapy alone for the palliation of hepatic metastases: Results of a Radiation Therapy Oncology Group randomized prospective trial. Int J Radiat Oncol Biol Phys. In press.

Patterns of Failure in Combined Chemotherapy and Radiotherapy for Limited Small Cell Lung Cancer: Southeastern Cancer Study Group Experience

Robert Birch,[1,*] George A. Omura,[1] F. Anthony Greco,[2] and Carlos A. Perez[3]

ABSTRACT—In this analysis, we compare the patterns of failure in the first 12 months for 660 eligible patients randomized to two Southeastern Cancer Study Group (SECSG) protocols for limited extent, small cell carcinoma of the lung between 1978 and 1985. In each protocol, a different schedule of radiotherapy was given in conjunction with combination chemotherapy and was compared with combination chemotherapy alone. In protocol 78 LUN 328, radiotherapy was given between courses of chemotherapy, and in protocol LUN 81343, it was given simultaneously. The rates of local failure, either as an initial or subsequent site, in the first 12 months were significantly lower when thoracic irradiation was given than when it was not ($P<.01$). When the 2 radiotherapy arms were compared, there were no significant differences in the rates of local failure alone, but a smaller proportion of patients developed both local failure and distant metastases ($P<.01$) when simultaneous radiotherapy was administered. Survival on all 4 arms was similar during the first 2 years of patient study. After 2 years, both radiotherapy regimens showed a trend toward improved survival compared with the combination drug alone (cyclophosphamide, doxorubicin, vincristine) arms. On both protocols, survival from 12 months was significantly longer for those with local control at 12 months than for those who did not show local control.—NCI Monogr 6:265–270, 1988.

Approximately 20% of all newly diagnosed carcinoma of the lung is SCLC. Broadly speaking, SCLC is staged in two categories: limited extent disease, in which disease is limited to one hemithorax with or without involvement of ipsilateral or contralateral supraclavicular lymph nodes, and extensive disease, which encompasses all disease greater than limited disease (*1,2*).

Limited extent SCLC is a radiosensitive and chemosensitive tumor, and recent reports have shown more complete responses and improved intrathoracic control when chemotherapy and RT are combined, often, however, without corresponding increases in survival.

Perez et al. (*3*) reported preliminary results of an SECSG protocol indicating improved intrathoracic control for patients treated with combination chemotherapy and RT. In this report, we update that study and compare the outcome with the results obtained from a recently completed follow-up study.

PATIENTS AND METHODS

Between 1978 and 1985, the SECSG completed 2 major studies on limited extent SCLC in which chemotherapy with CAV was compared to identical chemotherapy with the addition of thoracic RT as described below. In this analysis, we compare the patterns of failure, including both initial and subsequent sites, during the 12 months following entry of patients to the study.

Protocol 78 LUN 328.—This protocol was begun in 1978 and was based on a pilot study done at Vanderbilt University (*4*).

Patients were eligible for the study if they had limited extent SCLC with no disease in the contralateral supraclavicular lymph nodes, or had had a surgical resection of tumor with no measurable disease at the time of entry. All diagnoses were confirmed either by biopsy at the time of bronchoscopy, by sputum cytology, bronchial washings, or, occasionally, by lymph node biopsy. All patients gave informed consent.

Patients were required to have negative bone, brain, and liver scans as well as negative bone marrow biopsy. All scans were repeated at the conclusion of each phase of treatment and also when indicated to establish relapse. Any patient with low Karnofsky performance status (<30%) or with an uncontrolled second primary tumor was ineligible for the study. Likewise, any patient who had received previous chemotherapy or RT or who had ipsilateral pleural effusions was excluded.

Induction chemotherapy (iv) included 6 cycles of 1,000 mg cyclophosphamide/m^2 (maximum dose 2,000 mg) and 40 mg doxorubicin/m^2 (maximum dose, 100 mg) on day 1 every 3 weeks, and 1 mg vincristine/m^2 (maximum dose, 2 mg) on day 1, then weekly in the first cycle and every 3 weeks thereafter.

After induction chemotherapy (~15 wk), patients with progressive disease were to receive 1 g methotrexate/m^2 by iv infusion for approximately 1 hour, followed by leucovorin rescue (20 mg/m^2 by iv push) 24 hours after the methotrexate and then 10 mg by mouth every 6 hours for 12 doses. The regimen was administered on days 1 and 14 of a 28-day cycle. Patients with nonprogressive or responding disease received 3 cycles of 200 mg etoposide/m^2 iv on days 1 and 8 concurrently with 280 mg hexamethylmelamine/m^2 orally for the first 14 days of a 28-day

---

ABBREVIATIONS: SCLC = small cell lung cancer; RT = radiotherapy (irradiation); SECSG = Southeastern Cancer Study Group; CAV = cyclophosphamide, doxorubicin, vincristine.

[1] Southeastern Cancer Study Group Statistical Center, LBW Tumor Institute, University of Alabama at Birmingham.

[2] Vanderbilt University, Nashville, TN.

[3] Washington University, St. Louis, MO.

[*] *Reprint requests:* Robert Birch, Ph.D., Biological Therapy Institute, Hospital Dr., Franklin, TN 37064.

cycle and 50 mg pyridoxine orally twice daily administered throughout the 3 cycles.

Patients randomized to thoracic RT received it in a split course during weeks 5, 8, and 11 in between, but not simultaneously, with drug administration. A dose of 1,500 rad in 5 fractions was administered in weeks 5 and 8 with an additional 1,000 rad in 4 fractions in week 11 for a total dose of 4,000 rad. All patients received brain RT with 1,200 rad given in 5 fractions in weeks 5, 8, and 11 for a total dose of 3,600 rad. If patients had no measurable disease at the initiation of protocol therapy, RT was given to the hilar and mediastinal structures.

The schema for the protocol is shown in figure 1.

Protocol LUN 81343.—This was begun while 78 LUN 328 was still in an early follow-up phase. This protocol was built upon promising results of a study during which Ihde et al. (*5*) used RT simultaneously with chemotherapy rather than sequentially. Simultaneous treatment was evaluated with the hope that better local control would be provided.

The eligibility criteria for LUN 81343 were identical to those for 78 LUN 328 with the exception that patients with ipsilateral pleural effusions could be entered in the study as could those with contralateral supraclavicular lymph nodes, provided that all disease could be encompassed in a single radiotherapy field.

Patients received 6 cycles of induction chemotherapy with CAV as described for 78 LUN 328. Complete and partial responders after induction were randomized to receive either intensification or no further therapy. Intensification therapy consisted of 100 mg etoposide/m^2 by iv infusion over 30–60 minutes on days 1–4 and 20 mg cisplatin/m^2 in 100 ml normal saline over 15 minutes on days 1–4. Patients were to receive 2 cycles of intensification given 4 weeks apart.

Patients randomized to thoracic RT received 3,000 rad to the primary tumor in 10 fractions given simultaneously with chemotherapy starting on day 1 over a 2-week period. Patients then received an additional 1,500 rad in 5 fractions with the third cycle of chemotherapy. As in 78 LUN 328, patients with no measurable disease received RT to the hilar and mediastinal structures. If patients had extensive ipsilateral pleural effusions, as much of the entire lung as necessary could be irradiated to include the primary tumor and effusions.

Cranial RT, although initially mandated, was later given only to patients in complete remission at the end of induction at the discretion of the treating physician.

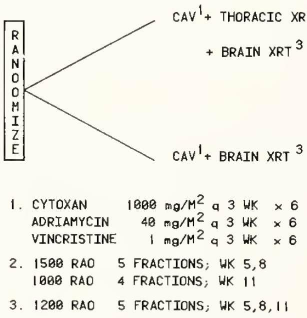

RANDOMIZE
CAV¹+ THORACIC XRT²
+ BRAIN XRT³
CAV¹+ BRAIN XRT³
1. CYTOXAN 1000 mg/M² q 3 WK × 6
ADRIAMYCIN 40 mg/M² q 3 WK × 6
VINCRISTINE 1 mg/M² q 3 WK × 6
2. 1500 RAD 5 FRACTIONS; WK 5,8
1000 RAD 4 FRACTIONS; WK 11
3. 1200 RAD 5 FRACTIONS; WK 5,8,11

FIGURE 1.—Schema for induction regimens on protocol 78 LUN 328.

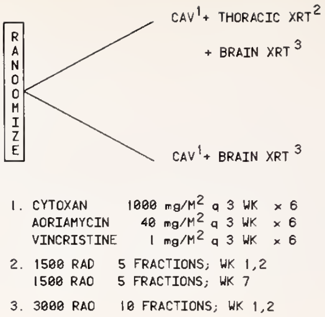

RANDOMIZE
CAV¹+ THORACIC XRT²
+ BRAIN XRT³
CAV¹+ BRAIN XRT³
1. CYTOXAN 1000 mg/M² q 3 WK × 6
ADRIAMYCIN 40 mg/M² q 3 WK × 6
VINCRISTINE 1 mg/M² q 3 WK × 6
2. 1500 RAD 5 FRACTIONS; WK 1,2
1500 RAD 5 FRACTIONS; WK 7
3. 3000 RAD 10 FRACTIONS; WK 1,2

FIGURE 2.—Schema for induction regimens on protocol LUN 81343.

The schema for protocol LUN 81343 is shown in figure 2.

Definitions of response.—The usual criteria for response as defined by the SECSG were used for both studies: Complete response was defined as the complete disappearance of all objective evidence of disease; partial remission was defined as a decrease of 50% or more in the sums of products of perpendicular diameters of the lesions that could be evaluated; no progression was defined as a decrease of less than 50% or an increase of less than 25% in the sum of the products of diameters of measurable lesions; progressive disease was defined as an increase of more than 25% in the sum of the products of the diameters of measurable lesions or new lesions appearing during the study.

Statistical methods.—For the purposes of comparison of the 2 studies, the proportion and types of relapses occurring in the first 12 months after the initiation of protocol therapy are used. We compared all proportions using an adjusted chi-square test (*6*). Survival curves were plotted according to the Kaplan-Meier technique (*7*) and compared by a log-rank test (*8*).

RESULTS

Protocol 78 LUN 328

A total of 304 patients were randomized to induction on protocol 78 LUN 328, of whom 291 were eligible for study. Reasons for ineligibility were extensive disease (2 CAV, 4 CAV plus RT); incorrect histology (3 CAV, 1 CAV plus RT); prior RT (2 CAV plus RT); and 1 patient not a candidate for RT (1 CAV). A total of 142 were eligible to receive CAV alone and 149 to receive CAV plus RT.

Protocol LUN 81343

A total of 386 patients, of whom 369 were eligible, were randomized to CAV with or without simultaneous thoracic RT. One hundred fifty-six were randomized to CAV and simultaneous thoracic RT, and 230 received CAV alone. Both groups received brain RT at the discretion of the investigator. Reasons for ineligibility were extensive disease (4 CAV plus RT, 4 CAV); incorrect histology on pathology review (2 CAV plus RT, 4 CAV); and 3 patients randomized to receive CAV plus RT who subsequently were found to be unable to receive RT. A total of 147 patients were eligible to receive CAV plus RT and 222 patients to receive CAV alone. The reason for this apparent imbalance is that accrual was stopped to the induction randomization after interim analysis showed significant differences with regard to toxicity, and all subsequent patients were entered on CAV alone until sufficient patients were randomized on the intensification phase.

Patterns of Failure

Table 1 shows patterns of failure in the first 12 months of therapy for all eligible patients on protocols 78 LUN 328 and LUN 81343. The body of the table is broken down into two sections corresponding to patients for whom we had sufficient information to evaluate for response to induction and those for whom we did not. Results in this paper are presented for all eligible patients. Our rationale for the structure of the table is to allow a direct comparison for 78 LUN 328 with previously reported data (*3,9*).

The local failure rates for the CAV with or without brain RT arms on the 2 protocols were similar; on 78 LUN 328, 60 of 142 (42%) of the patients had only local failure in the first 12 months compared with 89 of 222 (40%) on protocol LUN 81343 ($P=.76$). If one includes those patients who developed distant metastases as well as local failure, the numbers rise to 64 of 142 (45%) on 78 LUN 328 and 110 of 222 (50%) on LUN 81343 ($P=.47$). The rates of distant metastases alone were 19 of 142 (13%) on 78 LUN 328 compared with 19 of 222 (9%) on LUN 81343 ($P=.20$). The patterns of failure remain comparable between the 2 populations whether one considers all eligible patients, all patients at risk for 12 months, or all those for whom we had sufficient information for evaluation.

A question of greater interest lies in a comparison of the two arms receiving thoracic RT. For patients treated with RT given in the "sandwich" protocol 78 LUN 328, the rate of patients showing local progression only was 34 of 149 (23%) compared with 27 of 147 (18%) when RT is given simultaneously as in LUN 81343 ($P=.42$). If one includes those who progressed both locally and with distant metastases, the proportions progressing become 52 of 149 (35%) for 78 LUN 328 and 32 of 147 (22%) for LUN 81343 ($P=.02$). When we considered those patients who failed in the first year with only distant metastases, the rates are 16 of 149 (11%) for 78 LUN 328 and 17 of 147 (12%) for LUN 81343 ($P=.96$). Thus the overall rate of distant metastases for the RT arms are similar, i.e., 34 of 149 (23%) for 78 LUN 328 and 22 of 147 (15%) for LUN 81343 ($P=.11$), but a smaller proportion on protocol LUN 81343 have developed local progression. Once again, the results are similar for other subsets on the data.

On each protocol, the proportion of patients showing no local failure at 12 months was significantly higher if thoracic RT was given. On 78 LUN 328, the rates with only local failure were 60 of 142 (42%) without thoracic RT compared with 34 of 144 (23%) if thoracic RT was given ($P<.01$). Likewise for those with local failure with or without distant metastases, the proportions were 64 of 142 (45%) when no thoracic RT was given and 52 of 149 (35%) if it was ($P=.10$). On protocol LUN 81343, the corresponding results were 89 of 222 (40%) versus 27 of 147 (18%; $P<.01$) and 110 of 222 (50%) versus 32 of 147 (22%), respectively ($P<.01$).

Survival

Figure 3 shows overall survival from the initiation of therapy for each of the arms of protocols 78 LUN 328 and LUN 81343. The thoracic RT arm of 78 LUN 328 is significantly different from the no thoracic RT arm of that protocol, as we observed by the log-rank test used to compare the two curves ($P=.04$). All other differences are not statistically significant. Median survivals are 53.7 and 45.9 weeks for the patients receiving and not receiving thoracic RT, respectively, on 78 LUN 328. Median survival for those receiving thoracic RT on LUN 81343 was 45.4 versus 50.8 weeks for those who did not. Of the 149 patients on the thoracic RT arm of 78 LUN 328, 122 (82%) died of all

TABLE 1.—Patterns of progression in first 12 mo after initiation of therapy for eligible patients on protocol 78 LUN 328 and LUN 81343

		Treatment							
		CAV ± brain RT				CAV ± brain RT + thoracic RT			
		78 LUN 328		LUN 81343		78 LUN 328		LUN 81343	
Judgment on patient response to induction[a]	Progression of disease	No. of patients	%	No. of patients	%	No. of patients	%	No. of patients	%
Ineligible for evaluation	Local	11	7	0	0	8	5	0	2
	Distant	3	2	1	1	2	1	0	0
	Local and distant	0	0	0	0	3	2	0	0
	None at 12 mo	2	1	0	0	5	3	3	2
	Censored at <12 mo[b]	24	17	28	13	17	11	30	20
	Not evaluated	40	28	29	13	35	23	33	22
Eligible for evaluation	Local	49	35	89	40	26	17	27	18
	Distant	16	11	18	8	14	9	17	12
	Local and distant	4	3	21	9	15	10	5	3
	None at 12 mo	17	12	40	18	44	30	39	27
	Censored at <12 mo[b]	16	11	25	11	15	10	26	18
	Evaluated	102	72	193	87	114	77	114	78
	Total No. of eligible patients	142		222		149		147	

[a] Judgment was based on whether we had sufficient information to evaluate patient response.

[b] For detailed explanation, *see* table 2.

Table 2.—Reasons for censoring of information in first 12 mo for eligible patients on protocols 78 LUN 328 and LUN 81343

Judgment on patient response to induction[a]	Reasons	No. of patients and treatment			
		CAV±RT		CAV± brain RT+thoracic RT	
		78 LUN 328	LUN 81343	78 LUN 328	LUN 81343
Ineligible for evaluation	On induction				
	Early death not related to cancer	5	1	3	7
	No data available for progression	13	8	11	6
	Toxic deaths	2	3	0	2
	Lost to follow-up	4	16	3	15
	Not evaluated	24	28	17	30
Eligible for evaluation	To induction				
	Toxic deaths	0	1	0	6
	Early death not related to cancer	9	6	6	8
	No data available for progression	5	5	6	3
	Lost to follow-up	2	5	3	5
	Still in active follow-up	0	8	0	4
	Evaluated	16	25	15	26
	Total No. censored at <12 mo	40	53	32	56

[a] *See* footnote *a*, table 1.

causes as have 115 of 142 (81%) of those who received no thoracic RT. The equivalent figures are 91 of 147 (62%) and 144 of 222 (65%), respectively, for LUN 81343.

Of interest also is the effect of thoracic RT on long-term survival. On 78 LUN 328, the Kaplan-Meier estimate of 2-year survival was 24% for those receiving thoracic RT versus 16% for those who did not ($P=.05$). Equivalent figures for LUN 81343 were 29% and 21% ($P=.12$). At the 2-year point, all arms had a minimum of 10 patients at risk.

One possible explanation of these different survival rates at the 2-year point may be the different rates of local failure observed at the 12-month point. Figures 4 and 5 show survival from 12 months according to the presence or absence of local failure at 12 months. It is clear that in both protocols, thoracic control rather than the regimen used is associated with prolonged survival. This result is consistent with analyses presented earlier for non-small cell lung cancer (*9*). On 78 LUN 328, 30 of 34 patients with no local control are dead compared with 42 of 65 with local control. Median survivals from the beginning of therapy are 64.8 weeks for those with no local control and 129.9 weeks for those with local control. The equivalent figures on protocol LUN 81343 include 22 of 34 patients with no local control who are dead compared with 20 of 58 patients who had local control. Median survivals from the initiation of protocol therapy were 69.3 and 127.6 weeks, respectively.

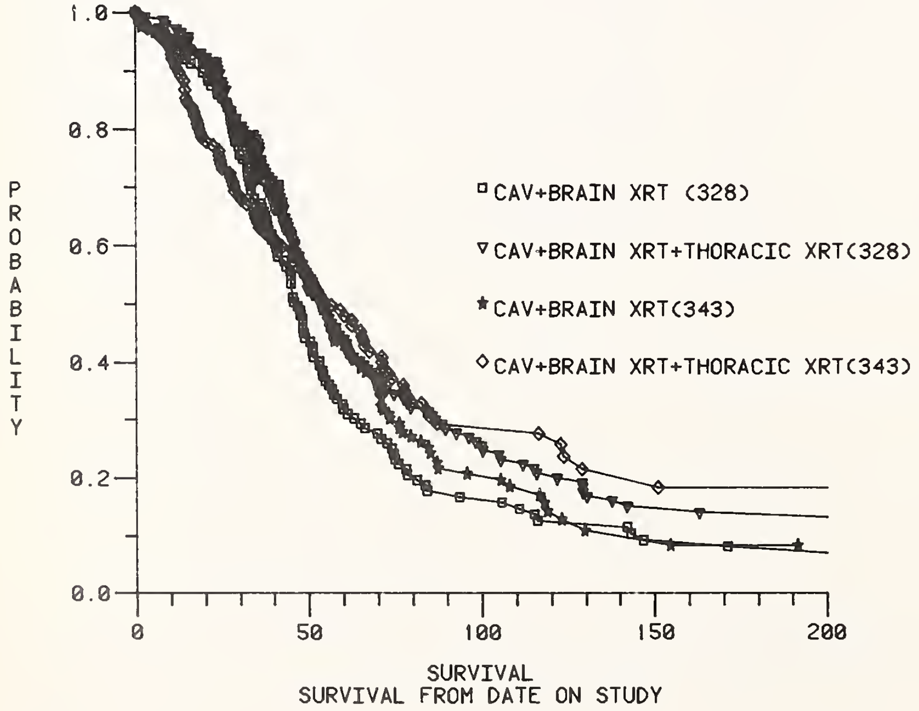


Figure 3.—Overall survival curves for induction regimens on protocols 78 LUN 328 and LUN 81343.

DISCUSSION

In this analysis, we compared rates of local failure using 2 RT schedules. Variations in the 2 schedules include both timing (sandwich vs. simultaneous) and dose (4,000 vs. 4,500 rad) with an apparent improvement in local control for simultaneous RT and the higher dose. Choi and Carey (*10*) have indicated that the rate of local control may be related to dose, suggesting that doses in the range of 5,000 rad or higher are needed to improve local control. The optimal timing of the RT is also a controversial issue. Goodman and co-workers (*11*) administered 4–6 cycles of CAV and etoposide followed by thoracic RT (5,000 rad in 25 fractions over 5 wk) to 36 of 38 patients in complete remission at the time of irradiation. Four patients had thoracic recurrences and 8 survived longer than 3 years. In contrast, investigators at the National Cancer Institute gave chest RT (4,000 rad, 15 fractions, 3 wk) beginning simultaneously with initiation of chemotherapy (cyclophosphamide, methotrexate, and vincristine) and showed an improved rate of complete remission (81% vs. 42%), median survival (16 vs. 12 mo), and 2-year, disease-free survival (35% vs. 13%).

Two other parameters are of major importance in the comparison of these regimens, i.e., toxicity and overall survival. Using the simultaneous RT schedule, we observed a 15% incidence of grades 3 and 4 esophagitis compared with 9% when the sandwich schedule was used. Other toxicities were similar. On the simultaneous RT arm, there were 6 toxic deaths (5%) compared with 3 (2%) on the sandwich schedule.

With regard to overall survival, both regimens show improvements in long-term (2 yr or longer) survival with rates around 25% compared with 16% for chemotherapy alone, but the difference was significant only for protocol 78 LUN 328. However, in the short term, there is an adverse effect on survival when the simultaneous regimen is used compared with chemotherapy alone or the sandwich regimen. Other reports of combined RT and chemotherapy have indicated either no significant differences in survival (*12,13*) or slight advantages in favor of the RT arms (*3,5*).

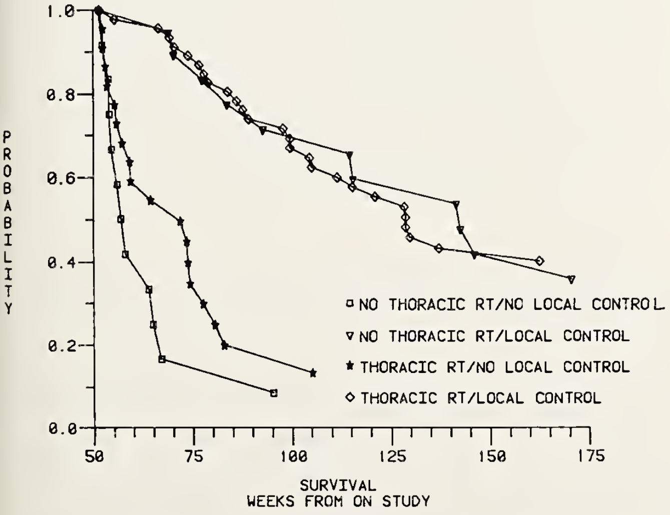


FIGURE 4.—Survival from 12 mo by local control at 12 mo for protocol 78 LUN 328.

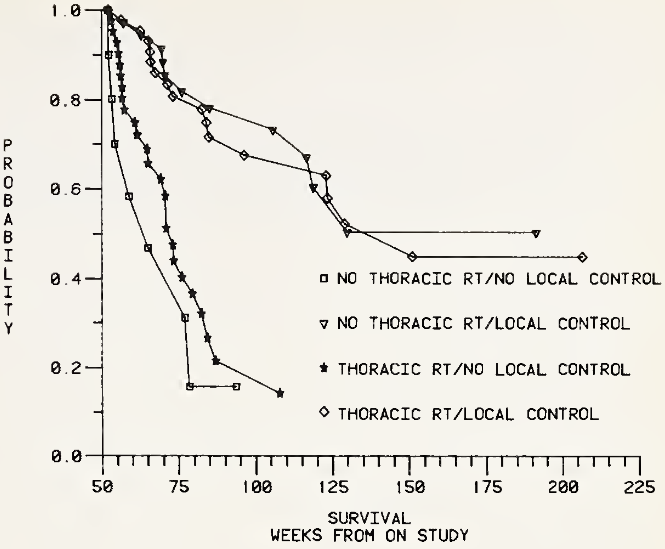


FIGURE 5.—Survival for 12 mo by local control at 12 mo for protocol LUN 81343.

Thus we conclude that both combinations of chemotherapy and RT are effective in improving local control of the tumor and 2-year survival compared with chemotherapy alone. The simultaneous schedule is associated with increased local control of the tumor at 12 months in contrast to the sandwich schedule, but the 2-year Kaplan-Meier estimate of survival is identical on the 2 regimens. The simultaneous regimen is also associated with increased esophagitis and treatment-related mortality. At this juncture, the sandwich schedule of RT appears to have the greater therapeutic index of the 2 RT schedules for combined modality therapy with CAV. However, the greatest need for physicians treating patients with SCLC is to have improved systemic chemotherapy regimens with which to combine the RT.

REFERENCES

(*1*) IHDE DC, HANSEN HH: Staging procedures and prognostic factors in small cell carcinoma of the lung. *In* Small Cell Lung Cancer (Greco FA, Oldham RK, Bunn PA, eds). New York: Grune & Stratton, 1981, pp 261–283.

(*2*) OSTERLIND K, IHDE DC, ETTINGER DS, et al: Staging and prognostic factors in small cell carcinoma of the lung. Cancer Treat Rep 67:3–9, 1983.

(*3*) PEREZ CA, EINHORN L, OLDHAM RT, et al: Randomized trial of radiotherapy to the thorax in limited small cell carcinoma of the lung treated with multiagent chemotherapy and elective irradiation. A preliminary report. J Clin Oncol 2:1200–1208, 1984.

(*4*) OLDHAM RK, GRECO F: Small cell lung cancer: A curable disease. Cancer Chemother Pharmacol 4:173–177, 1980.

(*5*) IHDE DC, BUNN PA, LICHTER AS, et al: Randomized trial of chemotherapy with or without adjuvant chest irradiation in limited stage small cell lung cancer. *In* Adjuvant Therapy of Cancer (Jones SE, Salmon SE, eds), vol IV. Orlando, FL: Grune & Stratton, 1984, pp 147–155.

(*6*) ARMITAGE P: Statistical Methods in Medical Research. Oxford, England: Blackwell, 1971, pp 131–135.

(*7*) Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481, 1958.

(*8*) Mantel N: Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 50:163–170, 1966.

(*9*) Perez CA, Bauer M, Edelstein S, et al: Impact of tumor control on survival in carcinoma of the lung treated with irradiation. Int J Radiat Oncol Biol Phys 12:539–547, 1986.

(*10*) Choi CH, Carey RN: Small cell anaplastic carcinoma of lung: Reappraisal of current management. Cancer 37: 2651–2657, 1976.

(*11*) Goodman GE, Miller TP, Manning MM, et al: Treatment of small cell lung cancer with VP16, vincristine, doxorubicin (Adriamycin), cyclophosphamide (EVAC) and high dose chest radiotherapy. J Clin Oncol 2:483–488, 1983.

(*12*) Stevens E, Einhorn L, Rohn R: Treatment of limited small cell lung cancer. Proc Am Assoc Cancer Res ASCO 20:435, 1979.

(*13*) Dombernowsky P, Hansen HH, Hansen M, et al: Treatment of small cell anaplastic bronchogenic carcinoma. Results from 1000 randomized trials. *In* World Conference on Lung Cancer. Copenhagen: Excerpta Medica, 1980, p 149.

(*14*) Souhami RL, Geddes DM, Spiro SG, et al: Radiotherapy in small cell cancer of the lung treated with combination chemotherapy: A controlled trial. Br Med J 288:1643–1646, 1984.

Use of Hemibody Irradiation as a Non-cross-resistant Agent in Combination With Systematic Chemotherapy in Small Cell Lung Cancer[1]

A. R. Belch,[2,*] R. C. Urtasun,[2] D. Bodnar,[2] B. Kinney,[2] and R. Amy[3]

ABSTRACT—Previously, investigators at this institute have studied the use of upper hemibody irradiation as a consolidation agent following combination chemotherapy for small cell lung cancer. There was no improvement in survival compared to that in the group treated with chemotherapy alone. In our present pilot study, we are investigating the toxicity and efficacy of using hemibody irradiation (HBI) as a non-cross-resistant agent early in a program of alternating chemotherapy consisting of six treatment cycles. Toxicity due to the combined effects of chemotherapy (cisplatin and etoposide plus cyclophosphamide, doxorubicin, and vincristine) plus HBI is reported. The HBI was given at a dose of 1,000 cGy in four fractions for limited disease or as a single 800-cGy dose for extensive disease. Bone marrow suppression following HBI necessitated a subsequent delay in the chemotherapy cycle or dose reduction in 55% of the 33 patients. Six patients developed diffuse interstitial pneumonitis following chemotherapy and HBI; 3 have died, and in 2 of these, the etiology was opportunistic infection. In our previous studies utilizing HBI either alone or as consolidation therapy after induction chemotherapy, a low incidence of lung toxicity occurred. This increased incidence suggests a possible drug-radiation interaction when HBI is used as an alternating agent with doxorubicin and cisplatin.—NCI Monogr 6:271-274, 1988.

Despite recent therapeutic gains in the management of SCLC, a treatment plateau has been reached, demonstrating the need for improved strategies for the use of irradiation and chemotherapeutic agents (*1*). Many theories related to drug resistance are postulated to explain the inability of chemotherapy and irradiation to improve the cure rate for SCLC. A mathematical model relating the drug sensitivity of a tumor to its spontaneous mutation rate has suggested that the early use of alternating, non-cross-resistant drug combinations should lead to greater success (*2*). The clinical testing of this hypothesis has been difficult because it is hard to demonstrate that drug combinations in alternating schedules are equally effective and also non-cross-resistant. We and others have demonstrated that HBI is an effective agent in SCLC when given as a single agent with no chemotherapeutic drugs (*3,4*). Following this observation, the role of HBI as consolidation therapy after combination chemotherapy was evaluated in our institution in a pilot study (*5*). More recently, it is undergoing evaluation as a non-cross-resistant agent used much earlier in the induction program of alternating combination chemotherapy. We now report the results obtained in both of these studies.

METHODS

Patients with all stages of SCLC from a population of 1.3 million are referred to the Cross Cancer Institute, the only radiotherapy/chemotherapy facility for the northern part of Alberta and contiguous areas. Two investigational study protocols have been evaluated. The only exclusion criteria for patients are age >72 years or a Karnofsky performance status <50%. Staging procedures consist of computed tomography scan of the abdomen and thorax; liver, brain, and bone isotope scans; and bone marrow biopsy. After these procedures, patients are stratified by stage. Localized disease is defined as disease within the chest or affecting ipsilateral supraclavicular nodes; extensive disease is defined as proven malignant pleural or pericardial effusions and/or disease beyond the chest. Staging procedures are repeated at the completion of the induction treatment to assess tumor response.

Study I

The role of HBI as a consolidating agent was evaluated in a sequential prospective study from 1981 to 1985. Treatment design consisted of an induction phase of four cycles of chemotherapy at 3-week intervals; cyclophosphamide (1,000 mg/m^2) and doxorubicin (45 mg/m^2) were given on day 1 and etoposide (80 mg/m^2) was given on days 1–3. Immediately following the third cycle of chemotherapy, the primary lesion in the chest was irradiated to a total tumor dose of 5,000 cGy in 25 fractions in an overall time of 5 weeks, with the spinal cord receiving <3,500 cGy. The fourth cycle of chemotherapy was not delayed by the radiotherapy. In patients whose disease was localized, doxorubicin was not administered in the third and fourth cycles, to prevent treatment-induced esophagitis. For patients with extensive disease, however, the total radiation dose was lowered to 3,500 cGy and the doxorubicin was included in the third and fourth cycles of chemotherapy. No separate

ABBREVIATIONS: SCLC = small cell lung cancer; HBI = hemibody irradiation; UHBI = upper hemibody irradiation; LHBI = lower hemibody irradiation.

---

[1] Supported by grant No. 5230 from the Alberta Heritage Savings Trust Fund: Applied Cancer Research.

[2] Departments of Medical Oncology (*A. R. Belch*) and Radiation Oncology (*R. C. Urtasun, D. Bodnar,* and *B. Kinney*), Cross Cancer Institute, Edmonton, Alberta, Canada.

[3] Department of Pathology, University of Alberta, Edmonton, Canada.

* *Reprint requests:* A. R. Belch, M.D., Department of Medical Oncology, Cross Cancer Institute, 11560 University Ave., Edmonton, Alberta T6G 1Z2, Canada.

prophylactic cranial irradiation was given because this site would be included in the HBI field.

Initially, 4 weeks after completion of induction, all responders received 1,000 cGy in four equal fractions to the upper hemibody, with the lungs shielded to receive 600 cGy corrected for lung density; 6–8 weeks later, provided there was complete bone marrow recovery, they received 1,000 cGy in four fractions to the lower hemibody. This consolidation therapy was not followed by any maintenance treatment. After evaluation of 35 cases, it was apparent that virtually all of the failures occurred in the field of the UHBI; consequently, the LHBI consolidation was discontinued and the UHBI dose was increased to 2,000 cGy in eight equal fractions. The lungs were shielded to receive 600 cGy corrected for lung density, and the previous irradiated volume in the chest, including the spinal cord, was completely shielded. Both kidneys were shielded to receive <1,000 cGy, and the eyes and salivary glands were shielded anteriorly. After 37 patients were enrolled in this treatment design, the HBI consolidation treatment was not used for the subsequent 42 patients; only combination chemotherapy and local chest irradiation were given. Since no HBI was given, prophylactic cranial irradiation (3,500 cGy in 15 fractions over 21 days) was given at the completion of the induction therapy in all responding patients in this last treatment group.

Study II

Although HBI is an effective single agent, when used as a consolidating agent, it has not improved overall treatment results. Recently though, very impressive survival data for SCLC patients with limited disease were reported with use of six cycles of combination chemotherapy with cisplatin and etoposide alternating with cyclophosphamide, doxorubicin, and vincristine when thoracic irradiation was included concurrently at the beginning of treatment (*6*).

Consequently, in November 1985, we initiated a pilot study of the utilization of UHBI as an additional non-cross-resistant agent early in the induction phase of all stages of SCLC and employed the same alternating chemotherapy program as Murray et al. (*6*). In this way, we hoped to gain further tumor cell kill and to reduce the opportunity for acquired resistance.

The eligibility, staging criteria, and re-evaluation procedures are the same as those for study I. The treatment scheme, which is shown in table 1, consists of six cycles of treatment at 3-week intervals. The chemotherapy combination of cyclophosphamide (1,000 mg/m^2), doxorubicin (50 mg/m^2), and vincristine (2 mg) is alternated with etoposide (100 mg/m^2 daily×3) and cisplatin (25 mg/m^2 daily×3). The chemotherapy doses are reduced to 75% if the WBC count is 2,500–3,000/mm^3 and the platelet count is >100,000/mm^3 at the time of the next cycle. For more profound myelosuppression, chemotherapy is delayed until improvement occurs. In limited disease, UHBI of 1,000 cGy in four equal fractions with no lung shielding is given as the second treatment cycle, 21 days after the first cycle of chemotherapy. In extensive disease, UHBI of 800 cGy in a single dose is delivered with shielding of the lungs to receive 600 cGy (uncorrected for lung density), using a transmission block, but the lung primary site is not shielded. The UHBI treatment volume in both limited and extensive disease is modified from our earlier studies so that the skull and humeri are excluded. The primary chest lesion in limited disease is irradiated beginning at the first chemotherapy cycle. The total tumor dose is 4,000 cGy in 20 equal fractions for 4 weeks with an interruption during the week that HBI is delivered, and the spinal cord dose is calculated to be <3,500 cGy. Prophylactic brain irradiation (3,500 cGy in 15 fractions over 21 days) is administered to complete responders at the end of the last cycle of induction therapy because the modified HBI treatment volume does not include the head.

TABLE 1.—Pilot protocol[a]

Cycle 1	2	3	4	5	6
		Localized disease[b]			
CDDP, E	UHBI	CAV	CDDP, E	CAV	CDDP, E
		Extensive disease[c]			
CDDP, E	UHBI	CAV	CDDP, E	CAV	CDDP, E

[a] CDDP = cisplatin, E = etoposide, C = cyclophosphamide, A = doxorubicin, V = vincristine.

[b] Local irradiation to primary lesion begins with 1st cycle of chemotherapy and continues for 4 wk at dose of 4,000 cGy with break during week UHBI is delivered. Dose of UHBI = 1,000 cGy in 4 fractions.

[c] Prophylactic brain irradiation is delivered to complete responders at end of last cycle of induction because modified UHBI treatment volume does not include head. Dose of UHBI = 800 cGy in 1 dose.

RESULTS AND DISCUSSION

Study I

As shown in table 2, the median survival times for patients with limited disease in these 3 treatment groups were 56.5 weeks (UHBI/LHBI), 62 weeks (UHBI only), and 62.5 weeks (no HBI). The 2-year survival results for limited disease for the first 2 treatment groups are 23% and 18%, respectively. Insufficient time has elapsed for 2-year survival figures for the consolidation group with no HBI. We observed no conversions from partial to complete response after consolidation HBI, and we conclude that intensive chemotherapy for four cycles followed by HBI did not improve the overall survival rate beyond that achieved with induction chemotherapy and local irradiation without consolidation HBI. Based on these data, we cannot recommend the use of HBI as a consolidation technique in SCLC.

Neurological toxicity has been reported in patients who achieve long-term survival, probably due to prophylactic brain irradiation and perhaps accentuated by chemotherapeutic agents, but this complication has not been observed in this study nor in our previous protocol employing HBI (*7,8*).

Study II

Since November 1985, 39 patients have been entered in this study; 20 had limited disease, and 19 had extensive disease. Of the 21 patients entered >18 weeks ago, 19 have completed the protocol. Complete response was seen in 83% of the patients with limited disease and 60% of those with extensive disease; the remainder had partial response.

Table 2.—Treatment outcome of consolidation HBI

Treatment	N	Median survival (wk)		2-yr survival	
		Localized disease	Extensive disease	Localized disease	Extensive disease
Chemotherapy + local irradiation + UHBI/LHBI (1,000 cGy/4 fractions)	35	56.5	42.5	23%	0
Chemotherapy + local irradiation + UHBI only (2,000 cGy/8 fractions)	37	62	39.5	18%	6.5%
Chemotherapy + local irradiation only	42	62.5	37	[a]	[a]

[a] Data not yet available.

It is not possible to assess the impact of this treatment on survival because there was insufficient duration of follow-up.

TOXICITY

The HBI portion of this program produced moderate myelosuppression requiring modification of the subsequent cycle of chemotherapy treatment in most cases. Of the 33 patients who received HBI followed by at least one subsequent cycle of chemotherapy, 16 experienced an average delay of 2 weeks (range, 1–5) before the next treatment cycle could be given; and 10 of the 16 required a chemotherapy dosage reduction to 75% for the third cycle, due to grade 2 myelosuppression (Radiation Therapy Oncology Group scale; WBC count $<3{,}000/\text{mm}^3$). Virtually all of the adjustments in time and dose occurred in the third cycle, immediately following the HBI, with no delays or chemotherapy dose modification in the ensuing cycles. This is in contrast to a previous group of 25 patients treated with the same chemotherapy protocol plus local irradiation of the primary tumor but without HBI; these patients required no chemotherapy dose reduction or cycle delay. In our study, 4 of 29 patients had moderate dysphagia or odynophagia, which produced esophagitis requiring a puree or liquid diet. Each of these patients had limited disease and had received radical thoracic irradiation in addition to the HBI. The symptoms resolved within 1–3 weeks and no sequelae were observed.

Lung toxicity (table 3) has become a major problem in our study. Six patients have developed diffuse, bilateral interstitial pneumonitis following chemotherapy and HBI. A fatal diffuse radiation pneumonitis developed in 1 patient with limited disease 14 weeks after HBI and following completion of five of the six cycles of therapy. Another patient with limited disease developed biopsy-proven, nonfatal diffuse bilateral radiation pneumonitis 28 weeks after the HBI and following completion of all cycles of therapy. This patient improved with steroids. A bilateral diffuse fatal cytomegalovirus pneumonia proven by autopsy and culture occurred after the third cycle of therapy in a patient with extensive disease, and a fatal diffuse bilateral opportunistic pneumonia (organism uncertain) occurred 3 months after completion of induction therapy in a patient with limited disease. In 2 additional patients, diffuse interstitial pneumonitis occurred 20 and 28 weeks after HBI and after completion of six cycles of therapy, but these patients improved with steroid treatment.

Table 3.—Bilateral diffuse interstitial pneumonitis

Patient No.	Stage[a]	Onset after HBI (wk)	No. of therapy cycles	Cause		Outcome
				Infection	Radiation	
1	LD	14	5	No	Yes	Fatal
2	ED	6	3	Cytomegalovirus	No	Fatal
3	LD	24	6	Suspected	Possible	Fatal
4	LD	28	6	No	Yes	Improved
5	ED	20	6	No	Yes	Improved
6	LD	28	6	No	Yes	Improved

[a] LD = localized disease, ED = extensive disease.

This high rate of lung complications is disturbing. In our original study of UHBI given as a single fraction of 600 cGy to both lungs, with no chemotherapy, the incidence of symptomatic diffuse interstitial pneumonitis was 13%, but no cases were fatal (*4*). The incidence was reduced to none in 72 patients who received fractionated HBI with lung shielding to 600 cGy (*5*). In the current pilot study, the lung dose is either 1,000 cGy in four equal fractions in patients with limited disease or a single 600-cGy dose in patients with extensive disease; yet, the pneumonitis rate is unacceptably high. We believe this must be related to an interaction of the HBI and chemotherapy drugs on the lung parenchyma. The major differences between this present study and our previous study using fractionated HBI for consolidation are the incorporation of HBI early in the treatment program followed by further cycles of chemotherapy, the increase of the lung dose to 1,000 cGy in four equal fractions in limited disease, and the use of cisplatin. The total dose of doxorubicin in our new study is only 100 mg/m^2, slightly *less* than that given in study I. Symptomatic diffuse interstitial pneumonitis has not been reported to date by other groups using the same alternating drug combination program without HBI, but a 25% incidence has been reported for an intense chemotherapy protocol of cyclophosphamide, methotrexate, and etoposide as induction therapy and 1-year maintenance therapy (*9*). The prolonged exposure to methotrexate was implicated as a possible causative factor in that program.

Recently, a much lower incidence of pneumonitis (5%) was reported for a detailed, prolonged combination chemotherapy program (*10*). These authors suggested that initial pulmonary function tests could predict post-treatment pulmonary complications, but this was not apparent in our patients. Although it is clear that doxorubicin can potentiate radiation damage and also reactivate a prior episode of radiation-induced pneumonitis, less is known about the normal tissue damage produced by the interaction of radiation and cisplatin (*11*). Several investigators have shown that cisplatin and radiation act synergistically in model systems and that the drug may inhibit repair of sublethal radiation injury (*12–14*). We speculate that such mechanisms of interaction between cisplatin and radiation may be the etiology of the increased incidence of pneumonitis in our study. In an attempt to reduce the unacceptably high incidence of pneumonitis, we now plan to shield the contralateral lung *entirely* from the HBI in all future patients in this study.

REFERENCES

(*1*) OLDHAM RK: Small cell lung cancer: A curable disease. Clin Oncol 4:153–159, 1985.

(*2*) GOLDIE JH, COLDMAN AJ, GADAUSKAS GA: Rationale for the use of alternating non-cross-resistant chemotherapy. Cancer Treat Rep 66:439–449, 1982.

(*3*) EICHHORN HJ, HUTTNER J, DALLUGE KH, et al: Preliminary report on "one-time" and high dose irradiation of the upper and lower half body in patients with small cell lung cancer. Int J Radiat Oncol Biol Phys 9:1459–1465, 1983.

(*4*) URTASUN RC, BELCH AR, MCKINNON S, et al: Small cell lung cancer: Initial treatment with sequential hemibody irradiation vs 3-drug systemic chemotherapy. Br J Cancer 46:228–235, 1982.

(*5*) URTASUN RC, BELCH AR, BODNAR D, et al: Radiation as a non-cross-resistant systemic agent: Experience with hemibody and total body irradiation in patients with small cell lung cancer. Cancer Treat Symp 2:41–47, 1985.

(*6*) MURRAY N, HADZIC E, SHAH A, et al: (Abstr) Alternating chemotherapy and thoracic radiotherapy with concurrent cisplatinum for limited stage small cell carcinoma of the lung. J Clin Oncol 3:214, 1984.

(*7*) LOOPER JD, EINHORN LH, GARCIA SA, et al: (Abstr) Severe neurologic problems following successful therapy for small cell lung cancer. J Clin Oncol 3:231, 1984.

(*8*) LEE JS, LEE YY, UMSAWASDI T, et al: (Abstr) Neurotoxicity in long term survivors of small cell lung cancer. J Clin Oncol 3:220, 1984.

(*9*) ZIMMERMAN MS, RUCKDESCHEL JC, HUSSAIN M: Chemotherapy induced interstitial pneumonitis during treatment of small cell lung cancer. J Clin Oncol 2:396–404, 1984.

(*10*) BROOKS BJ, SEIFTER EJ, WALSH TE, et al: Pulmonary toxicity with combined modality therapy for limited stage small cell lung cancer. J Clin Oncol 4:200–209, 1986.

(*11*) MCINERNEY DP, BULLIMORE J: Reactivation of radiation pneumonitis by Adriamycin. Br J Radiol 50:224–227, 1977.

(*12*) KALLMAN RF, SUTHERLAND RM: Combined modalities. Cancer Treat Symp 1:103–110, 1984.

(*13*) DRITSCHILO A, PIRO AJ, KELMAN AD: The effect of *cis*-platinum on the repair of radiation damage in plateau phase Chinese hamster (V-78) cells. Int J Radiat Oncol Biol Phys 5:1369–1372, 1979.

(*14*) YUHAS JM, TARLETON AE, CUOL F: Tumor line dependent interactions of irradiation and *cis*-diamminedichloroplatinum in the multicellular tumor spheroid system. Int J Radiat Oncol Biol Phys 5:1373–1375, 1979.

Ten-year Results of Randomized Trial Comparing Radiotherapy and Concomitant Bleomycin to Radiotherapy Alone in Epidermoid Carcinomas of the Oropharynx: Experience of the European Organization for Research and Treatment of Cancer[1]

F. Eschwege,* H. Sancho-Garnier, J. P. Gerard, M. Madelain, A. DeSaulty, A. Jortay, and Y. Cachin[1]

ABSTRACT—**This trial of treatment for head and neck carcinoma was initiated in 1973 by the European Organization for Research and Treatment of Cancer. Its purpose was to investigate the value of single-agent chemotherapy with bleomycin (BLM) given during the course of a conventional treatment by external radiotherapy (RT) compared to treatment by external RT alone. In this randomized study, we compared treatment results in 2 groups of patients with squamous cell carcinoma of the oropharynx (T2, T3, and T4; International Union Against Cancer classification). One group of 92 patients was treated by RT at the prescribed dose of 70 Gy. The other group of 107 patients received radiation according to the same protocol and simultaneously received im injection of BLM at a dose of 15 mg twice a week, 2 hours prior to the session of RT, for a total dose of 150 mg in 5 weeks. The occurrence of local toxic effects (i.e., mucositis and epidermatitis) was significantly greater in the RT-BLM group (RT-BLM, 72%, vs. RT, 21%). Primary tumor response 6 weeks after completion of RT was the same in both arms of the study (RT, 68%, vs. RT-BLM, 67%). The 6-year survival rate was 24% (RT-BLM) versus 22% (RT). Long-term analysis (10 yr) is given.—NCI Monogr 6:275-278, 1988.**

Radiotherapy is the usual treatment for patients with epidermoid carcinomas of the oropharynx, but their long-term prognosis remains poor, with a survival rate ≤30% at 5 years (*1-3*). Preliminary clinical studies of patients with carcinomas of the head and neck have suggested a cumulative antitumor effect of BLM when it is given in conjunction with RT (*4,5*).

The aim of this trial was to compare BLM administered in conjunction with RT to RT alone with regard to disease-free survival and overall survival in patients with carcinoma of the oropharynx. A first report was previously published (*6*); we are presenting here the results after 10 years.

PATIENTS AND METHODS

All patients with histologically proven squamous cell carcinoma of the base of the tongue, tonsillar fossa, or posterior wall and soft palate who had tumors that were >2 cm or infiltrating were included in the trial regardless of the status of the nodes. Patients were excluded from this trial if they had been previously treated or if they had distant metastasis; a second primary tumor other than skin carcinoma; poor general status; bone marrow depression; kidney failure; chronic pulmonary disease; or diabetes mellitus.

Patients were randomly stratified by institution and were allocated to 1 of the 2 following groups:

1) *RT alone.*—The primary tumor and the clinically involved lymph nodes were to receive a dose of 70 Gy for 7-8.5 weeks, while clinically uninvolved nodes received 50-55 Gy for 5-6 weeks.
2) *RT with concomitant BLM.*—The same protocol of RT was used; 15 mg of BLM was administered im or iv twice a week from the start of RT for 5 weeks. Each injection of BLM was to be given 2 hours prior to the session of RT. The total dose was 150 mg.

The first end point of this study was tumor regression. At 6 weeks after completion of RT, tumor regression was evaluated (100%, >50%, none, or growth), and the residual volumes of the primary tumor and the palpable nodes were compared to the measured initial volumes. Immediate toxicity was recorded. The second end point was comparison of survival curves and late effects in the 2 treatment groups.

Data management and statistical analysis were accomplished by the statistical unit of IGR. The randomization was stratified according to institution and was balanced after every 4 patients within each institution.

RESULTS

Patients

From April 1973 to December 1974, 224 patients from 15 centers were registered. Two centers are excluded from the final analysis because they only provided 1 patient each,

---

ABBREVIATIONS: BLM = bleomycin; RT = radiotherapy; IGR = Institut Gustave-Roussy; EORTC = European Organization for Research and Treatment of Cancer.

[1] This study was conducted by the Head and Neck Cooperative Group of the European Organization for Research and Treatment of Cancer. The following members participated in the study: Institut Gustave-Roussy, Villejuif, France (*F. Eschwege, H. Sancho-Garnier,* and *Y. Cachin*); Institut Jules Bordet, Bruxelles, Belgium (*A. Jortay*); Centre Oscar Lambret, Lille, France (*M. Madelain*); Hôpital Régional, Lille, France (*A. DeSaulty*); and Centre Léon Bérard, Lyon, France (*J. P. Gerard*). Other participating institutions include: Istituto di Oncologia, Torino, Italy (*F. Badellino*); Centre Antoine Lacassagne, Nice, France (*F. Demand*); Hôpital Tenon, Paris, France (*S. Hamel*); Centre Paoli-Calmette, Marseille, France (*M. Jausseran*); H. N. O. Klinik Universitat, Berlin, Germany (*H. R. Nitze*); Antoni van Leeuwenhoek Ziekenhuis, Amsterdam, The Netherlands (*G. B. Snow*); Hôpital St. André, Bordeaux, France (*L. Traissac*); and St. Radboud Ziekenhuis, Nijmegen, The Netherlands (*P. Van Den Broek*).

* *Reprint requests:* F. Eschwege, M.D., Institut Gustave-Roussy, 39 a 53, rue Camille Desmoulins, 94805 Villejuif Cedex, France.

and 25 other patients are also excluded for different reasons; these patients were balanced within the 2 treatment groups. Thus, 199 patients were evaluated, 92 in the group with RT alone and 107 in the group receiving RT and BLM.

Table 1 shows the distribution of the main prognostic factors by treatment; the prognostic factors are sex, age, initial tumor site, tumor extent by number of sites, tissue involvement, and status of nodes. The 2 treatment groups are comparable with regard to the main prognostic factors.

Treatment

Radiotherapy

The average total dose was 64 Gy ± 2 SD in the group receiving RT alone and 63 Gy ± 1.70 SD in the group receiving BLM. This difference is not significant, but the average duration of RT was significantly longer ($P=.01$) in the BLM group. This difference is due to the more frequent delays during the course of RT, which were associated with mucosal toxicity experienced by the group receiving BLM (table 2). Protocol violations varied among institutions. In the group receiving BLM, either drug doses have been reduced or the course of RT has been delayed. These changes in treatment have been discussed in a previous report (*6*).

Bleomycin

Only 64% of the patients have received ≥150 mg of BLM; for the other patients, local reactions have led to discontination of BLM before completion of RT.

TABLE 1.—Distribution of cases by prognostic factors and treatment groups[a]

Factor	RT ($n=92$)	RT-BLM ($n=107$)	Total
Sex			
Male	87	94	91%
Female	5	13	
Mean age ± SD (yr)	57±0.6	57±0.5	
Site			
Tonsillar fossa	43	47	45%
Anterior pilar	—	1	0.5%
Posterior pilar	3	5	4%
Base of tongue	31	40	35%
Amygdaloglossal sulcus	7	4	5.5%
Soft palate	2	8	5%
Vallecula	3	1	2%
Oropharynx (unspecified)	3	1	2%
No. of sites			
1	27	37	32%
2	33	30	32%
>2	32	40	36%
Tissue involvement			
Mucosa	83	91	87%
Muscle	8	15	12%
Bone	1	1	1%
Cervical nodes			
N0	34	39	37%
Mobile nodes	29	31	30%
Fixed nodes	29	37	33%

[a] Unless otherwise specified, values = No. of patients.

TABLE 2.—Delay in RT due to toxic effects[a]

Treatment group	RT completed on schedule	RT postponed	RT stopped prior to completion
RT ($n=92$)	95%	5%	0%
RT-BLM ($n=107$)	70%	24%	6%

[a] $P<.01$.

Toxicity

The overall rate of toxic effects was 23% in the group receiving RT alone and 77% in the group receiving concomitant BLM ($P<.001$). This difference is mostly explained by the local reactions such as mucositis and epidermatitis, which were severe in only 21% of the patients treated by RT alone but in 71% of those receiving both RT and BLM. Although the occurrence of skin rash, pneumonopathy, hemorrhage, severe dysphagia, and fever was slightly more frequent in the BLM group, the difference between the groups was not significant. Weight loss and profound weakness were significantly more common in the groups receiving BLM because normal nutrition was impaired due to mucositis (table 3). Late toxic effects were evaluated only in the 37 patients treated at IGR.

Tumor Regression

Complete regression was observed in 66% of the patients, 69% in the RT group and 65% in the RT-BLM group. The difference between the regression rates of the 2 treatment groups is not significant even when the main prognostic factors are taken into consideration by adjustment techniques. There is likewise no significant difference between the regression rates for patients with clinically involved lymph nodes.

Survival

The actuarial survival curves shown in figure 1 indicate the following results:

1) There is no significant difference between the 2 groups (adjusted log-rank test).
2) The median duration of survival is 12 months in each group.

TABLE 3.—Toxic effects related to treatment[a]

Toxic effect	RT	RT-BLM	P value
Mucositis	12	50	
Epidermatitis	3	3	
Mucositis with epidermatitis	4	23	
Total	21%	71%	<.01
Skin rash	1	4	NS
Pneumonopathy	1	2	NS
Hemorrhage	3	3	NS
Severe dysphagia	0	1	NS
Fever	0	2	NS
Profound weakness	1	19	<.01
Weight loss	5	20	<.05
Total[b]	23%	77%	<.001

[a] Unless otherwise specified, values = No. of patients. NS = not significant.

[b] Some patients presented with 2 or 3 types of toxic effects.

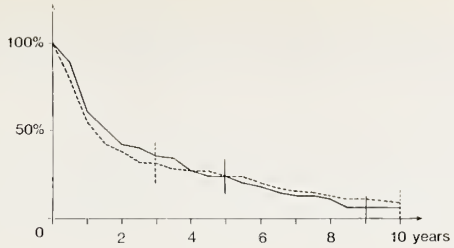


FIGURE 1.—Actuarial survival curves for 136 patients receiving RT (*solid line*) or RT-BLM (*broken line*) at IGR (Lyon and Lille).

3) The 5-year survival rates are 23% for the RT group and 22% for the RT-BLM group.
4) The disease-free survival is 22% for the RT group and 22% for the RT-BLM group.
5) The 10-year survival is <10% for the 2 arms of the study (IGR, Lyon and Lille, France).

DISCUSSION

This trial, which was the first one undertaken by the EORTC Head and Neck Cooperative Group, illustrated some of the difficulties encountered in conducting multicenter clinical trials. Insofar as these variations are mostly balanced between the 2 groups, we can expect that the results are not biased. The conclusions of this work are that concomitant use of BLM with RT as specified in the trial protocol results in more local and general complications but does not improve the survival of patients with oropharyngeal tumor.

We will not consider the comments previously reported concerning the methodological problems of this multicenter, randomized therapeutic trial.

Compliance with the protocol was not optimal; in particular, the treatment was not strictly in agreement with the protocol in 57% of the cases treated exclusively by RT and in 78% of the cases treated by combined RT-BLM, because of the occurrence of local adverse effects, especially severe radiomucositis.

Several investigators have encountered the same phenomenon, and they have emphasized the importance of local reactions. Shanta and Krishnamurthi (*7*) reported a randomized trial including 159 patients with squamous cell epithelioma of the oral cavity. In the group treated by a combination of RT and BLM with a therapeutic regimen identical to ours, the importance of mucosal reactions required a reduction in the total dose of radiation or, in some cases, a definitive interruption of the treatment.

Silverberg et al. (*8*) demonstrated a direct relationship between the intensity of local reactions and the total dose of BLM administered simultaneously with radiation. Doses of 15 mg of BLM administered twice weekly were barely effective, and radiation doses, which were >40–45 Gy, were lower than the 70-Gy dose in our initial protocol. These doses represent the average doses with the usual fractionation for head and neck tumors.

A different protocol was initiated by Fu et al. (*9*). During the first 2 weeks of RT, a combination of cyclophosphamide, vincristine, and BLM was administered. This was followed by BLM iv at a dose of 5 mg twice weekly after a rest period of 2 weeks. The treatment was stopped after 15 patients received it, because of severe local adverse effects.

Glick et al. (*10*) reported the same problem in patients treated with vincristine, BLM, and methotrexate simultaneously with RT. These severe reactions were not observed by Berdal et al. (*4*), who used a split technique, nor by Kapstad et al. (*11*) in their preoperative protocol, which delivered only 30 Gy in combination with a daily dose of 10 mg of BLM administered three times weekly.

Vermund et al. (*12*) reported the results of a randomized trial comparing 2 groups of patients, 1 treated with RT only and the other treated with a combination of RT and an im injection of 5 mg of BLM 1 hour before RT. Again, with this protocol, which was very similar to ours, RT had to be interrupted in the majority of the cases because of toxic effects.

None of the previously mentioned authors reported pulmonary toxicity.

The results of these studies show that local toxic effects make it impossible to use a standard dosage of radiation simultaneously with BLM. Few studies reported results concerning the late effects of RT.

For the subgroup of patients treated in IGR, there was no difference between the 2 groups in regard to the frequency of trismus, osteoradionecrosis, and cervical sclerosis. In addition, our preliminary results showed no difference between the 2 groups in tumoral and nodal regression and survival rates. Survival at ≥5 years remains identical for the whole group of patients and the subgroup treated in our institution. In the study of Kapstad et al. (*11*), there was no significant difference in the sterilization rate between the 2 groups with or without BLM (preoperative RT).

The results of Glick et al. (*10*) in 11 cases were promising on a short-term basis (73% complete regression) but were poor at 18 months (18% complete regression). In the randomized study conducted by Shanta and Krishnamurthi (*7*), results were more favorable in the RT-BLM group, with 77% complete regression versus 21% in the RT group. Results were also better for survival at 2 years (50% vs. 17%, respectively) and at 4 years (50% vs. 3%, respectively).

For Vermund et al. (*12*), the responses to the treatment and the data on disease progression were the same in the RT and RT-BLM arms. Survival rates at 2 years (RT, 64%±5% SE, vs. RT-BLM, 57%±5% SE) and 5 years (42%±5% vs. 38%±5%, respectively) were almost equal in both groups. Likewise, locoregional control was similar at 2 years (59%±5% vs. 57%±5%, respectively) and at 5 years (58%±5% vs. 53%±5%, respectively) even for the group receiving ≥100 mg of BLM. The results of Stefani et al. (*13*), Lo et al. (*14*), and Bezwoda et al. (*15*) have shown no differences in survival between the groups treated with RT alone or combined with chemotherapy.

Because of the existence of more active (*16*) but also more toxic drugs, further trials should be considered. These trials should be randomized and should include a sufficient number of patients to allow an accurate long-term analysis.

It does not appear to be possible to use radiation doses of 70–75 Gy in combination with an active and potentially toxic chemotherapy agent, and it seems necessary to consider new trials in which standard treatment would be compared to a combined option leading to identical toxic reactions.

CONCLUSIONS

The long-term results of the therapeutic trial on oropharyngeal carcinoma of the EORTC Head and Neck Cooperative Group confirm the preliminary results already reported: that there is no benefit in the combination of simultaneous BLM chemotherapy and RT versus RT only. The results reported elsewhere concerning new drugs and more active or hypothetically more efficient protocols should be confirmed by randomized studies comparing these options to the usual locoregional treatment.

REFERENCES

(*1*) Bataini JP, Bernier J, Jaulerry C, et al: Size dose relationships and local control of oro- and hypopharyngeal cancers treated by radiotherapy. *In* Progress in Radio Oncology (Karcher KH, Kogelnik HD, Reinartz G, eds), vol II. New York: Raven Press, 1982, pp 331–337.

(*2*) Fayos JV, Lampe I: Radiation therapy of carcinoma of the tonsillar region. Am J Roentgenol 111:85–94, 1971.

(*3*) Fletcher GH: Oral Cavity and Oropharynx. Textbook of Radiotherapy (Fletcher GH, ed), 2nd ed. Philadelphia: Lea & Febiger, 1975, pp 212–254.

(*4*) Berdal P, Iversen OH, Weyde R: Simultaneous intermittent bleomycin and radiological treatment of laryngeal cancer. Can J Otolaryngol 4:219–224, 1975.

(*5*) Rygard J, Hansen HS: Combined bleomycin and irradiation. Can J Otolaryngol 4:209–212, 1975.

(*6*) Cachin Y, Jortay A, Sancho H, et al: Preliminary results of a randomized EORTC study comparing radiotherapy and concomitant bleomycin to radiotherapy alone in epidermoid carcinomas of the oropharynx. Eur J Cancer 13:1389–1395, 1977.

(*7*) Shanta V, Krishnamurthi S: Combined therapy of oral cancer, bleomycin and radiation: A clinical trial. Clin Radiol 28:427–429, 1977.

(*8*) Silverberg IJ, Phillips TL, Fu KK, et al: Bleomycin with radiation therapy in the treatment of head and neck malignancies. Proc Am Assoc Cancer Res ASCO 17:279, 1976.

(*9*) Fu KK, Silverberg IJ, Phillips TL, et al: Combined radiotherapy and multidrug chemotherapy for advanced head and neck cancers. Results of a Radiation Therapy Oncology Group pilot study. Cancer Treat Rep 63:351–357, 1979.

(*10*) Glick JH, Fazekas JT, Davis LW, et al: Combination chemotherapy-radiotherapy for advanced inoperable head and neck cancer. A RTOG pilot study. Cancer Clin Trials 2:129–136, 1979.

(*11*) Kapstad B, Bang G, Reynaes S, et al: Combined preoperative treatment with cobalt and bleomycin in patients with head and neck carcinoma. A controlled clinical study. Int J Radiat Oncol Biol Phys 4:85–89, 1978.

(*12*) Vermund H, Kaalhus O, Winmer F, et al: Bleomycin and radiation therapy in squamous cell carcinoma of the upper digestive tract. A phase III clinical trial. Int J Radiat Oncol Biol Phys 11:1877–1886, 1985.

(*13*) Stefani S, Eells RW, Abbate J: Hydroxyurea and radiotherapy in head and neck cancer. Radiology 101:391–396, 1971.

(*14*) Lo T, Wiley AL, Ansfield FJ, et al: Combined radiation therapy and 5-FU for advanced squamous cell carcinoma of the oral cavity and oropharynx: A randomized study. Am J Roentgenol 126:229–235, 1976.

(*15*) Bezwoda WR, De Moor NG, Derman DP: Treatment of advanced head and neck cancer by means of radiation therapy plus chemotherapy. A randomized trial. Med Pediatr Oncol 6:353–358, 1979.

(*16*) Taylor SG IV, Murthy AK, Showel JL, et al: Improved contról in advanced head and neck cancer with simultaneous radiation and cisplatin/5-FU chemotherapy. Cancer Treat Rep 69:933–939, 1985.

Combined Modality Approach to Treatment of Malignant Gliomas—Re-evaluation of RTOG 7401/ECOG 1374 With Long-term Follow-up: A Joint Study of the Radiation Therapy Oncology Group and the Eastern Cooperative Oncology Group[1]

D. F. Nelson,[2] M. Diener-West,[3,4] J. Horton,[5] C. H. Chang,[6] D. Schoenfeld,[7] and J. S. Nelson[8,9,*]

ABSTRACT—The Radiation Therapy Oncology Group (RTOG) and the Eastern Cooperative Oncology Group (ECOG) conducted a phase III trial in patients with malignant gliomas to evaluate 4 treatment arms: 1) 60 Gy to the whole brain; 2) 60 Gy plus 10-Gy boost; 3) 60 Gy plus carmustine (BCNU); and 4) 60 Gy plus semustine plus dacarbazine. Between September 1974 and March 1979, 626 patients with malignant gliomas were treated on protocol RTOG 7401/ECOG 1374. Each institution chose a subset of the treatments to which the patients would be randomized. Patients were stratified according to subset and randomized to the 4 treatment arms. There were no differences in survival among treatment arms. For patients>60 years of age, the addition of chemotherapy to radiation therapy did not improve survival. For patients aged 40–60 years, there was a statistically significant increase in overall survival when BCNU was added to 60 Gy ($P<.01$), with an increase in 2-year survival from 8% to 23%. This beneficial effect of BCNU is apparent in both histological groups (astrocytoma with atypical or anaplastic foci and glioblastoma multiforme). Although few confirmatory autopsies are available, long-term survival in patients with astrocytomas with atypical and anaplastic foci who were treated with 60 Gy plus BCNU (5-yr survival, 22%) suggests no significant late CNS toxicity, compared to 60 Gy alone (5-yr survival, 15%). This is confirmed by comparable neurological function in long-term survivors.—NCI Monogr 6:279–284, 1988.

ABBREVIATIONS: RTOG = Radiation Therapy Oncology Group; ECOG = Eastern Cooperative Oncology Group; BCNU = carmustine; MeCCNU = semustine; DTIC = dacarbazine; BTCG = Brain Tumor Cooperative Group; XRT = radiation therapy; AAF = astrocytoma with atypical or anaplastic foci; GBM = glioblastoma multiforme; KPS = Karnofsky performance status.

The RTOG and the ECOG undertook the study RTOG 7401/ECOG 1374 to evaluate the efficacy of 1) an increased radiation dose of 70 Gy to the tumor, 2) addition of BCNU chemotherapy to 60 Gy, and 3) addition of combination chemotherapy of MeCCNU plus DTIC to 60 Gy, in comparison to conventional whole brain radiation therapy to a total dose of 60 Gy (*1*). The latter arm provided a common arm with the ongoing malignant glioma trial 7201 and the prior malignant glioma trial 6901 of the Brain Tumor Study Group (now BTCG). The BTCG 6901 trial had demonstrated that BCNU added to surgery and XRT increased the percent surviving 18 months from 4% to 19% (significant at $P<.01$ by chi-squared test), although it did not alter the median survival (*1*). The purpose of this paper is to report on the long-term follow-up of RTOG 7401/ECOG 1374, as long-term comparisons can be made with 94% of the patients deceased.

MATERIALS AND METHODS

Between September 1974 and March 1979, 626 patients with supratentorial malignant gliomas were treated by the RTOG and the ECOG in the phase III protocol RTOG 7401/ECOG 1374. Each institution chose a subset of treatments to which patients would be randomized. Patients had to have a biopsy-proven grade III or IV astrocytoma. These tumors have subsequently been recategorized as AAF and GBM by Nelson et al. (*2*) during central pathology review. Criteria for eligibility in the study were age <70 years; surgery within 4 weeks before starting treatment; and adequate renal, hepatic, and bone marrow functions.

After informed consent was obtained, patients were randomized to the subset of treatment options initially chosen by the institution. They were stratified accordingly and randomized to the following treatment arms: 1) 60-Gy whole brain radiation therapy; 2) 60 Gy plus 10-Gy boost; 3) 60 Gy plus BCNU at a dose of 80 mg/m^2 on days 1–3 and then 240 mg/m^2 iv every 6–8 weeks until March 1976, when toxicity led to dose modification to 80 mg/m^2 for 3 days every 8 weeks (60 Gy plus BCNU); and 4) 60 Gy plus MeCCNU plus DTIC (60 Gy plus MeCCNU plus DTIC). Before June 1975, DTIC was given at a dose of 175 mg/m^2 for 5 days every 4 weeks, and MeCCNU was given at a dose of 150 mg/m^2 orally every 8 weeks on day 5 of alternating courses of DTIC. Due to severe toxicity, the dose of DTIC was reduced to 150 mg/m^2 and MeCCNU to 125 mg/m^2 in June 1975. Chemotherapy was given for 2 years.

---

[1] Supported by Public Health Service grants CA-21661 and CA-32115 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Division of Radiation Oncology, Crozer-Chester Medical Center, Chester, PA.

[3] Radiation Therapy Oncology Group Statistical Unit, Philadelphia, PA.

[4] *Present address:* Department of Ophthalmology, The Johns Hopkins University, Baltimore, MD.

[5] Division of Oncology, Albany Medical College, NY.

[6] Department of Radiation Therapy, Columbia-Presbyterian Medical Center, New York, NY.

[7] Division of Biostatistics and Epidemiology, Sidney Farber Cancer Center, Harvard School of Public Health, Boston, MA.

[8] Department of Pathology, Washington University School of Medicine, St. Louis, MO.

[9] We thank Ms. Amy Berdann for preparing the manuscript.

* *Reprint requests:* Radiation Therapy Oncology Group Headquarters, 1101 Market St., 14th Floor, Philadelphia, PA 19107.

Supportive treatment consisted of corticosteroid therapy to control edema during the postoperative period and radiotherapy. A dose of 40–60 mg/day of prednisone or its equivalent was recommended for the first 4 weeks, under normal circumstances.

Initial assessment of the patient consisted of neurologic, medical, serum chemistry, and hematologic evaluations and determination of KPS (*3*) and neurological function (table 1). Pathology slides were submitted for central pathology review for confirmation and reclassification according to the classification of malignant gliomas reported by Nelson et al. (*2*) and others (*4–6*), in which GBM is distinguished from AAF by the presence of tumor necrosis in GBM.

After completion of treatment, patients were seen in follow-up every 3 months until death. In follow-up visits, patients were evaluated by assessment of neurological signs and symptoms, KPS, medications (especially corticosteroid dose), additional treatments, laboratory test results, x-rays, and nuclear scans. In addition to determination of survival rates, the quality of survival was also measured by changes in signs, symptoms, neurological function status, and KPS.

Patients were excluded from analysis if they did not meet the eligibility criteria, including histology; if they were withdrawn from the study before beginning XRT; or if they had insufficient documentation of eligibility.

Survival curves were estimated by the method of Kaplan and Meier (*7*). Comparisons of overall survival were made using the log-rank test (*8*). A previous Cox regression analysis of preliminary study results (*9*) identified age and histology as the two most important prognostic factors. In the present paper, overall survival comparisons were made among treatment groups, and the data were stratified by age (<40, 40–60, and >60 yr) and histology (AAF vs. GBM).

RESULTS

Between September 1974 and March 1979, 626 patients were entered in the study. Of these, 11 were canceled and 39 were ineligible. This left 576 eligible patients; 11 had no on-study information, and 27 had no follow-up information. The number of patients analyzed was 538: 141 in arm 1 (60 Gy); 103 in arm 2 (70 Gy); 156 in arm 3 (60 Gy plus BCNU); and 138 in arm 4 (60 Gy plus MeCCNU plus DTIC). Of the 538 eligible patients with follow-up, 94% are deceased. Follow-up ranged between 3 days and 11 years, with a median of 9.3 months.

TABLE 1.—Neurological function classification

Class	Description
I	Able to work or to perform normal activities Neurological findings minor or absent
II	Able to carry out normal activities with minimal difficulties Has neurological impairment that does not require nursing care or hospitalization
III	Seriously limited in performing normal activities Requires nursing care or hospitalization Confined to bed or wheelchair or has significant intellectual impairment
IV	Unable to perform even minimal normal activities Requires hospitalization and constant nursing care and feeding Unable to communicate or in coma

TABLE 2.—Patient characteristics by treatment[a]

Characteristic	60 Gy	70 Gy	60 Gy+ BCNU	60 Gy+ MeCCNU +DTIC
Age (yr)				
<40	18.4	15.5	12.8	15.2
40–59	56.0	59.3	57.1	57.3
>60	25.6	25.2	29.5	27.5
Unknown	0	0	0.6	0
KPS				
<70	43.3	42.7	46.1	45.0
>70	56.7	57.3	53.9	55.0
Extent of surgery				
Biopsy	21.3	21.4	18.6	16.6
Partial	59.6	55.4	51.3	56.5
Complete	14.9	20.4	22.4	23.3
Unknown	4.2	2.8	7.7	3.6
Pathology[b]				
AAF	13.5	16.5	17.9	16.7
GBM	76.6	76.7	73.7	71.7
Unknown	9.9	6.8	8.4	11.6
Neurological function				
I	37.6	31.1	34.6	27.5
II	35.5	41.7	41.0	47.1
III	24.8	21.4	21.8	23.2
Unknown	2.1	1.0	2.6	2.2
Total No. of patients	141	103	156	138

[a] Unless otherwise specified, values=% of patients.

[b] Central review.

The distributions of the major prognostic factors of age, KPS, and histology are given in table 2. The patient characteristics among the treatment groups are comparable.

Actuarial survival curves by treatment group are shown in figure 1. The median survival times are 9.3 months for 60 Gy; 8.2 months for 70 Gy; 9.7 months for 60 Gy plus BCNU; and 10.1 months for 60 Gy plus MeCCNU plus DTIC ($P=.39$). Survival by treatment groups differs by age. The survival curves for patients >60 years of age are superimposable ($P=.90$). For all patients >60 years of age, the overall median survival is 5.4 months; 1-year survival is 20.2% (range, 15.6–20.2 mo); and 2-year survival is 1.6% (range, 0–3.4%). The survival for patients aged 40–60 years is given in figure 2. The median survival times are 8.7, 8.2, 12.0, and 10.1 months for 60 Gy, 70 Gy, 60 Gy plus BCNU, and 60 Gy plus MeCCNU plus DTIC, respectively. The 2-year survival rates are 8.2%, 9.9%, 22.5%, and 17.0%, respectively. The 4-year survival rates are 2.4% for 60 Gy; 4.9% for 70 Gy; 6.3% for 60 Gy plus BCNU; and 9.3% for 60 Gy plus MeCCNU plus DTIC. For patients aged 40–60 years, there was a statistically significant difference ($P=.03$) in overall survival between treatment groups, with 308 patients in this age group. Separate analysis comparing 60 Gy with 60 Gy plus BCNU indicated improved survival

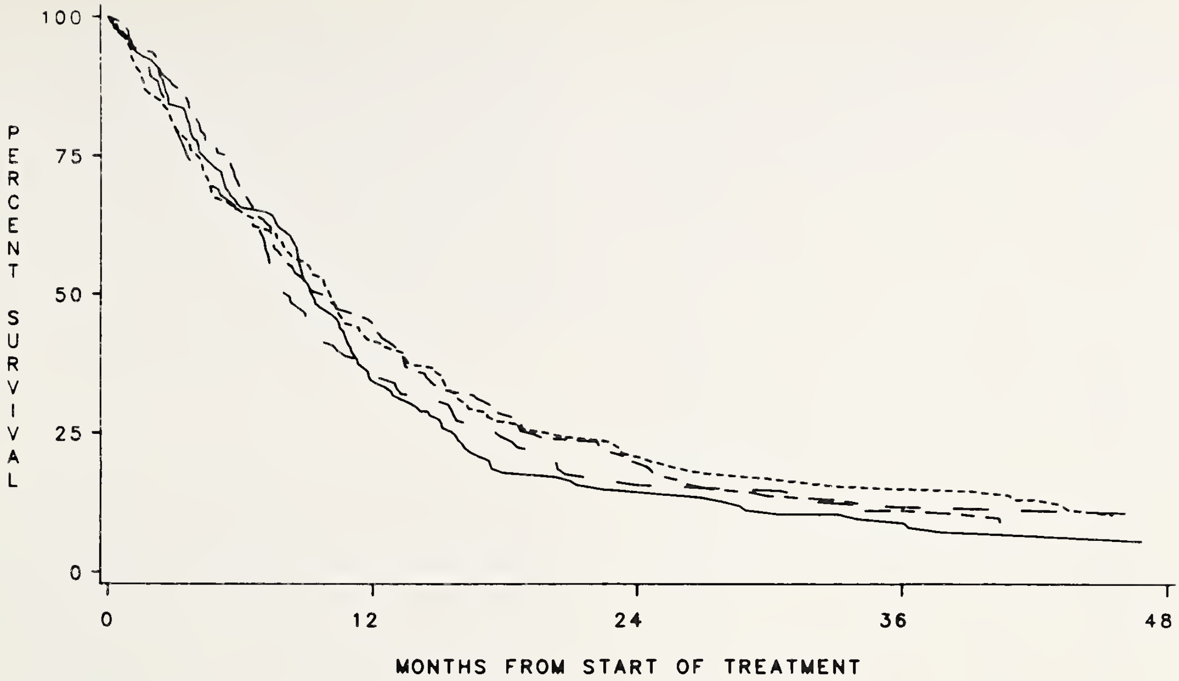


FIGURE 1.—Survival by treatment group. (—)=60 Gy; (— —)=70 Gy; (- —)=60 Gy+BCNU; and (----)=60 Gy+MeCCNU +DTIC.

with 60 Gy plus BCNU ($P < .01$), as evidenced by the difference in survival at 18 and 24 months.

The largest increase in percent surviving 2–5 years and in median survival is observed in the age group <40 years (fig. 3); in this group, the addition of chemotherapy increased the median survival of 21.3 months for 60 Gy to 29.9 months for 60 Gy plus BCNU and 33.1 months for 60 Gy plus MeCCNU plus DTIC. The addition of a 10-Gy boost to 60 Gy appeared to increase the median survival to 47.0 months, although this appears to be due to very long

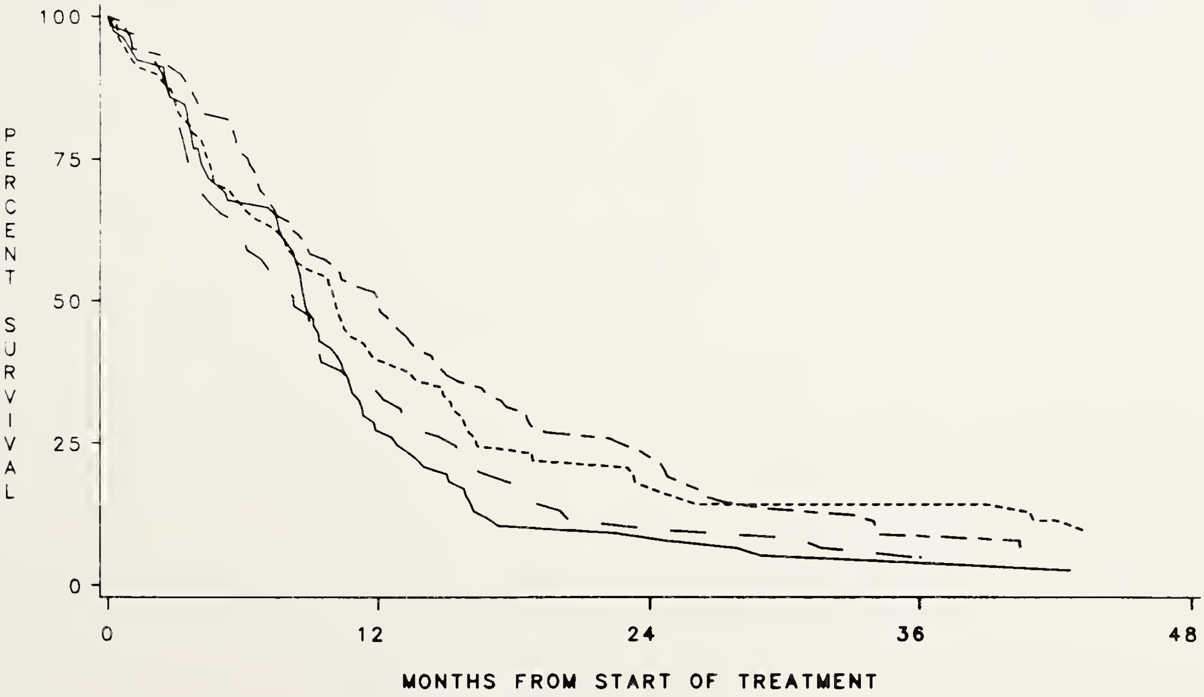


FIGURE 2.—Survival by treatment group for patients aged 40–60 yr. (—)=60 Gy; (— —)=70 Gy; (- —)=60 Gy+BCNU; and (----)=60 Gy+MeCCNU+DTIC.

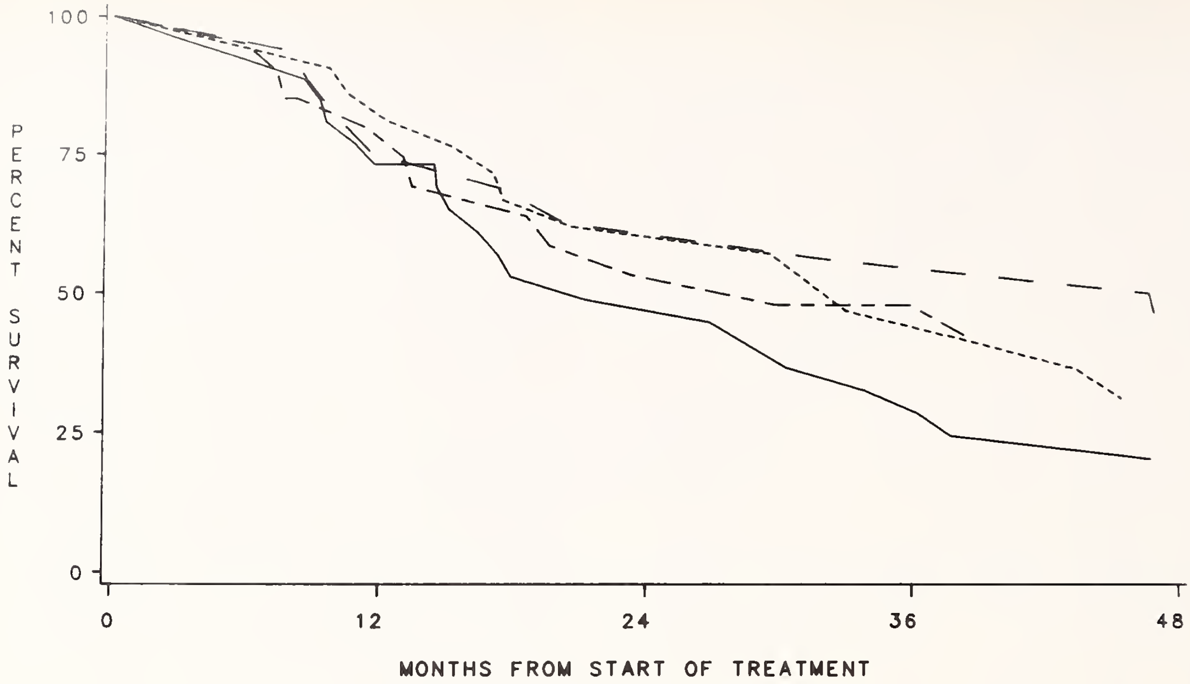


FIGURE 3.—Survival by treatment group for patients aged <40 yr. (—)=60 Gy; (— —)=70 Gy; (- —)=60 Gy+BCNU; and (---)=60 Gy+MeCCNU+DTIC.

survival observed in 6 AAF patients <40 years of age who were treated with 70 Gy. Survival rates for patients <40 years at 1, 2, 3, 4, and 5 years after 60 Gy (26 patients) are 73%, 47%, 28%, 20%, and 13%, respectively; after 60 Gy plus BCNU (20 patients), survival rates are 78%, 53%, 44%, 36%, and 34%, respectively. A comparison of the survival curves for these 2 treatment groups suggests better survival with the addition of BCNU (log-rank test; $P=.09$).

For all patients with GBM, the survival curves are almost superimposable ($P=.59$). The median survival times are

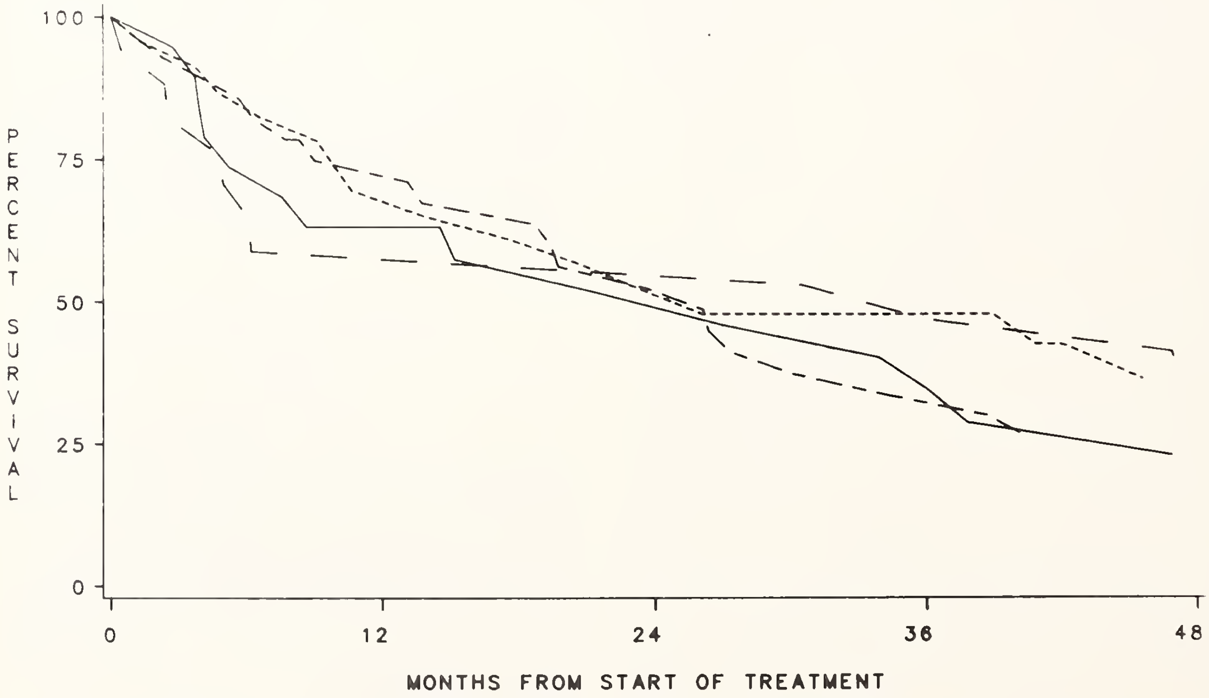


FIGURE 4.—Survival by treatment group for patients with AAF. (—)=60 Gy; (— —)=70 Gy; (- —)=60 Gy+BCNU; and (----)=60 Gy+MeCCNU+DTIC.

8.7 months for 60 Gy; 7.7 months for 70 Gy; 7.8 months for 60 Gy plus BCNU; and 9.2 months for 60 Gy plus MeCCNU plus DTIC. The respective survival rates at 2 years are 6.9%, 5.1%, 11.3%, and 12.2%. The survival by treatment for all patients with AAF is given in figure 4. There is no significant difference between the curves ($P=.81$). The median survival times are 26.9 months for 60 Gy; 35.9 months for 70 Gy; 26.1 months for 60 Gy plus BCNU; and 26.1 months for 60 Gy plus MeCCNU plus DTIC. The respective survival rates at 5 years are 15.3%, 26.9%, 21.8%, and 23.7%. The separate influence of age (<40 vs. 40-60 yr) and histology (AAF vs. GBM) on 18- and 24-month survival rates can be seen in table 3.

TOXICITY

The complications of treatment are given in table 4. Therapy with 60 Gy plus MeCCNU plus DTIC was substantially more toxic than 60 Gy plus BCNU. There were 5 patients with lethal thrombocytopenia/neutropenia: 1 who received 60 Gy plus BCNU on the original dose schedule and 4 who received 60 Gy plus MeCCNU plus DTIC (1 on the original dose schedule and 3 on the modified dose schedule). The functional status of patients after treatment was assessed by measuring the best neurological function class and KPS achieved during follow-up, relative to the baseline pretreatment neurological function and KPS. There was no difference between treatment arms. Overall, the neurological function improved in 34.4% of the patients, was stable in 55.9%, and worse in 9.7%. For patients with GBM, the neurological function improved in 33.2%, was stable in 56.3%, and worse in 10.5%. For patients with AAF, the neurological function improved in 37.2%, was stable in 54.7%, and worse in 8.1%. The results for KPS were similar, with no difference in KPS between treatment arms. Overall, KPS improved in 43.7% of the patients, was stable in 33.7%, and worse in 22.6%. For patients with GBM, KPS improved in 40.2%, was stable in 34.1%, and worse in 25.7%. For patients with AAF, KPS improved in 53.1%, was stable in 32.1%, and worse in 14.8%.

TABLE 3.—Percent survival at 18 and 24 mo for total population by treatment, histology, and age

	Survival (%)	
	18 mo	24 mo
All patients		
60 Gy	17.7	14.3
70 Gy	23.7	15.5
60 Gy + BCNU	28.2	19.5
60 Gy + MeCCNU + DTIC	26.7	20.6
All GBM		
60 Gy	10.4	6.9
70 Gy	14.5	5.1
60 Gy + BCNU	18.5	11.3
60 Gy + MeCCNU + DTIC	16.5	12.2
All AAF		
60 Gy	54.7	48.9
70 Gy	56.0	54.5
60 Gy + BCNU	64.1	51.7
60 Gy + MeCCNU + DTIC	60.3	51.1
<40 yr		
60 Gy	57.6	46.8
70 Gy	68.8	60.5
60 Gy + BCNU	65.6	52.7
60 Gy + MeCCNU + DTIC	67.2	60.1
40-60 yr		
60 Gy	10.3	8.2
70 Gy	16.4	9.9
60 Gy + BCNU	30.9	22.5
60 Gy + MeCCNU + DTIC	24.0	17.0

TABLE 4.—Percent complications by treatment

Complication	60 Gy	70 Gy	60 Gy + BCNU	60 Gy + MeCCNU + DTIC
Leukopenia				
Mild	2.1	2.9	18.0	13.8
Moderate	2.8	0	22.4	21.0
Severe	0	1.0	6.4	10.9
Life-threatening	0	0	3.8	5.8
Lethal	0	0	0.6	2.2
Thrombocytopenia				
Mild	0	1.9	17.9	13.8
Moderate	0.7	0	9.0	11.6
Severe	0.7	1.9	6.4	13.0
Life-threatening	0	0	3.8	9.4
Lethal	0	0	0.6	2.9
Intracranial pressure				
Mild	7.1	6.8	11.5	7.2
Moderate	8.5	1.9	8.3	4.3
Severe	0.7	1.0	3.2	3.6
Life-threatening	0.7	1.0	2.6	0
Lethal	0	0	0.6	0.7
Vomiting				
Mild	8.5	5.8	25.6	15.9
Moderate	5.0	1.0	14.1	30.4
Severe	0.7	0	5.1	8.7
Life-threatening	0	0	0	0.7
Lethal	0	0	0	0

The quality of life of the 39 patients alive at 4 years was assessed by neurological function class and KPS. Neurological function was stable or improved over baseline function for 82% of the patients; 69% had neurological function class I at 4 years. The KPS was stable or improved over baseline KPS for 76% of the patients, with 63% having a KPS of 90-100 at 4 years.

DISCUSSION

The absence of any significant difference in overall survival and in median survival between patients receiving 60 Gy or 60 Gy plus BCNU is similar to the results of the BTCG studies 6901 (*1*) and 7201 (*10*). In both studies, the addition of BCNU to radiation did produce a modest increase in 18-month survival (e.g., an increase of 4%-19% in study 6901). In our study, the only significant increase in survival by log-rank test was seen in the 40- to 60-year-old patients; the 2-year survival rate increased from 8.2% for 60 Gy to 22.5% for 60 Gy plus BCNU. If the 18-month survival rates had been used, the differences would have appeared larger (e.g., 10.3% for 60 Gy vs. 30.9% for 60 Gy plus BCNU). As can be seen in table 4, there generally is a greater difference between treatment arms at 18 months than at 24 months, as the curves appear to eventually come together with longer follow-up.

In BTCG study 7501, all patients received surgery and XRT. There was a statistically significant increase in survival (after adjusting for imbalance in prognostic factors among the treatment arms with the Cox regression) in the groups receiving BCNU or treatment with procarbazine added, compared to the group receiving treatment with only methylprednisolone added. However, it is interesting that there was no statistically significant difference in survival between the patients receiving surgery plus XRT plus methylprednisolone versus surgery plus XRT plus methylprednisolone plus BCNU (*11*). Some of the differences in treatment results among studies may be due to differences in the distribution pattern of prognostic factors among studies.

This update confirms the previously reported (*9*) increase in survival in the 40- to 60-year age group. For patients <40 years of age, the addition of chemotherapy increased the median survival as well as the percent surviving 2, 3, 4, and 5 years, although the numbers of patients were too small to allow statistically significant differences. Of interest is the marked increase in median survival to 47 months for the 70-Gy arm, which is double that for the 60-Gy arm, although the total patient number in this group is only 16.

The addition of BCNU or MeCCNU plus DTIC to 60 Gy did not alter the survival in the AAF group overall. For AAF patients <40 and 40–60 years old, the addition of chemotherapy increased survival modestly, albeit not significantly. A most important observation is that the addition of chemotherapy or 10-Gy boost did not worsen survival. This can be taken as indirect evidence that no significant late CNS complications such as brain necrosis are occurring. In neutron therapy trials, no differences were seen in overall survival between treatment arms of photon versus neutron or mixed beam therapy, but differences were observed when survival was analyzed by histology. Patients with AAF who were treated with neutrons had a lower survival rate than AAF patients treated with photons. Autopsies or second biopsies confirmed the presence of necrosis without gross tumor recurrence in the neutron arm (*12,13*). In the RTOG misonidazole trial (RTOG 79-18), there was no difference in survival between treatment arms for all patients or for patients with GBM. However, the survival rate for patients with AAF who were treated with 60 Gy plus BCNU plus misonidazole was much lower than that for control group AAF patients treated with 60 Gy plus BCNU (13.2 vs. 30.3 mo) (*14*), an observation also made by the BTCG (S. Green, personal communication, Sept. 1985).

The quality of life as measured by neurological function class also confirmed a lack of significant toxicity, as 77% and 83% of the patients alive at 4 years who had been treated with 60 Gy plus BCNU or 60 Gy, respectively, had neurological classification I and a KPS of 90–100. The acute toxicity of MeCCNU and DTIC was unacceptably high; there were 4 deaths (2.9%) from thrombocytopenia/neutropenia.

The lack of a significant increase in survival with 70 Gy is somewhat surprising, especially in view of the dose-response relationships observed by Walker et al. (*15*) at doses of 50, 55, and 60 Gy. This could be explained by geographic misses, as accurate tumor localization by computerized tomography scan was not available at the initiation of RTOG 7401/ECOG 1374.

In conclusion, the addition of BCNU to 60 Gy does appear to improve survival in patients <60 years of age. Since it does not alter survival in the older patient population, who are at a higher risk of developing complications, it is our recommendation that BCNU should not be given to patients >60 years old.

REFERENCES

(*1*) Walker MD, Alexander E, Hunt WE, et al: Evaluation of BCNU and/or radiotherapy in the treatment of anaplastic gliomas. J Neurosurg 49:333–343, 1978.

(*2*) Nelson JS, Tsukada Y, Schoenfeld D, et al: Necrosis as a prognostic criterion in malignant supratentorial astrocytic gliomas. Cancer 52:550–554, 1983.

(*3*) Karnofsky DA, Abelmann WH, Craver LF, et al: The use of nitrogen mustards in the palliative treatment of carcinoma. Cancer 1:634–656, 1948.

(*4*) Russel DC, Rubinstein LJ: Pathology of Tumors of the Nervous System, 4th ed. Baltimore: Williams and Wilkins, 1977.

(*5*) Burger PC, Vogel FS: Surgical Pathology of the Nervous System and Its Coverings. New York: Wiley, 1976.

(*6*) Burger PC: Malignant astrocytic neoplasms: Classification, pathologic anatomy, and response to treatment. Semin Oncol 13:16–26, 1986.

(*7*) Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481, 1958.

(*8*) Peto R, Peto J: Asymptotically efficient rank invariant test procedures. J R Stat Soc 135:185–207, 1972.

(*9*) Chang CH, Horton J, Schoenfeld D, et al: Comparison of postoperative radiotherapy and combined postoperative radiotherapy and chemotherapy in the multidisciplinary management of malignant gliomas. Cancer 57:997–1007, 1983.

(*10*) Walker MD, Green SB, Byar DP, et al: Randomized comparisons of radiotherapy nitrosoureas for the treatment of malignant glioma after surgery. N Engl J Med 303:1323–1329, 1980.

(*11*) Green SB, Byar DP, Walker MD, et al: Comparisons of carmustine, procarbazine, and high-dose methylprednisolone as additions to surgery and radiotherapy for the treatment of malignant glioma. Cancer Treat Rep 67: 121–132, 1983.

(*12*) Griffin TW, Davis R, Laramore G, et al: Fast neutron radiation therapy for glioblastoma multiforme: Results of an RTOG study. Am J Clin Oncol 6:661–667, 1983.

(*13*) Laramore GE, Diener-West M, Griffin TW, et al: RTOG randomized dose-searching study of mixed beam (neutron/photon) irradiation for high grade gliomas of the brain. Int J Radiat Oncol Biol Phys. In press.

(*14*) Nelson DF, Diener-West M, Weinstein AS, et al: A randomized comparison of misonidazole sensitized radiotherapy plus BCNU and radiotherapy plus BCNU for treatment of malignant glioma after surgery: Final report of an RTOG study. Int J Radiat Oncol Biol Phys 12:1793–1800, 1986.

(*15*) Walker MD, Strike TA, Sheline GE: An analysis of dose-effect relationship in the radiotherapy of malignant gliomas. Int J Radiat Oncol Biol Phys 5:1725–1731, 1979.

Pilot Study of Interaction of Radiation Therapy With Doxorubicin by Continuous Infusion

C. Julian Rosenthal* and Marvin Rotman[1]

ABSTRACT—Doxorubicin was initially administered alone by continuous infusion for 5 days every 3 weeks in escalating doses to 13 patients with advanced metastatic and/or recurrent malignancies. The maximum tolerable dosage was 13 mg/m^2 per day for 5 days. Kinetic data showed a steady level of 60 ng/ml for 4 days and a biphasic disappearance curve. Radiation therapy (150–200 cGy per session) was then administered in 5-day cycles, every 3 weeks, concomitantly with continuous infusion of doxorubicin (12 mg/m^2 per day) to 21 patients with various advanced unresectable recurrent or metastatic malignancies. Four of 9 patients with soft tissue sarcomas achieved complete response after a radiation dose of 2,206 ± 590 (SD) cGy and 3 had partial response; the median durations of the response were 142 ± 65 (SD) weeks for complete response and 28 ± 10 weeks for partial response. Of 4 patients with primary hepatoma, 2 achieved partial response after 1,290 ± 210 cGy. No response was seen in any of the 7 patients with adenocarcinoma of the gastrointestinal tract or breast. Complications of this regimen included moderate leukopenia and thrombocytopenia, mucositis, skin erythema, and decrease of the ventricular ejection fraction at a cumulative doxorubicin dose of 840 mg/m^2. We conclude that doxorubicin given by protracted infusion can be safely administered with concomitant radiation and appears to enhance the effects of radiation on most soft tissue sarcomas and on some hepatocellular carcinomas.—NCI Monogr 6:285–290, 1988.

Radiation therapy enhancement by chemotherapy agents has been described as additive or synergistic (*1*). The anthracyclines seem to be synergistic with radiation for both neoplastic cells and normal tissues. Consequently, this combination is potentially hazardous when delivered to large areas of vital structures. However, single pulses of doxorubicin in conventional doses (40–60 mg/m^2) administered concurrently with radiation therapy can be well tolerated (*2*).

In vitro studies (*3*) have shown that exposure of Yoshida ascites sarcoma cells to doxorubicin over increasing periods of time yielded increased cytotoxic effects against the tumor cells at proportionately lower doses. Furthermore, doxorubicin administered by continuous iv infusion was found (*4*) to have significantly less cardiotoxicity than by iv bolus. Based on these data, we postulated that concomitant administration of low-dose doxorubicin in continuous infusion with radiotherapy to locally advanced malignant lesions could further improve the therapeutic index.

At the time this study was started, there were no clinical data concerning the use of doxorubicin by continuous infusion nor any reported clinical experience with administration of this drug by protracted infusion concomitantly with radiation therapy; thus a phase I–II study was needed.

MATERIALS AND METHODS

Patient Population

Thirty-four patients were entered in this study over a period of 36 months; 13 received doxorubicin infusion alone, and 21 received doxorubicin infusion and concomitant irradiation to some of the measurable lesions. Seven of 13 patients also had measurable lesions that did not receive radiation therapy, thus permitting a comparison of the effect of combined modality treatment with that of doxorubicin infusion alone. Active infections, cardiac arrhythmia, cardiac failure, and bone marrow suppression (platelet count $<10^5/\mu l$ and WBC count $<4\times10^3/\mu l$) were contraindications for entering patients in this study. There were 15 females and 19 males varying in age between 16 and 75 years old. All patients had advanced metastatic and/or recurrent locoregional disease measurable by palpation, x-ray, or computerized tomography scan. All patients had pretreatment staging workup and had advanced metastatic or recurrent disease of various histologies and grades, with invasion of regional lymphatics or of neighboring structures (table 1). Several patients had been treated previously by bolus injections of doxorubicin or had received limited radiation therapy >3 months prior to entry in this study to lesions that subsequently showed an unequivocal increase in size.

Therapeutic Regimen

Doxorubicin was either dissolved in 1,000 ml of 5% dextrose solution in 0.5 *N* saline and delivered at a constant rate through silicone elastomer catheters (Deseret-Parke-Davis, Sandy, UT) inserted in the superior vena cava via the external jugular vein or was mixed with 60 ml of normal saline and delivered slowly into the subclavian vein through a Hickman catheter (*5*) connected to a battery-activated pump (Cormed, Medina, NY).

Initially in the phase I study, doxorubicin was given at a dosage of 9 mg/m^2 per day, which had been found to be appropriate in a prior study (*6*) when given by continuous intra-arterial infusion for 5 days and repeated every 3–4 weeks as soon as the bone marrow recovered. The dose was

---

ABBREVIATIONS: RIA = radioimmunoassay; STS = soft tissue sarcoma; PR = partial remission; CR = complete remission.

[1] Division of Medical Oncology (*C. J. Rosenthal*) and Department of Radiation Oncology (*M. Rotman*), State University of New York–Health Science Center at Brooklyn.

* *Reprint requests:* C. Julian Rosenthal, M.D., State University of New York–Health Science Center at Brooklyn, 450 Clarkson Ave., Box 55, Brooklyn, NY 11203.

TABLE 1.—Effects of concomitant radiotherapy plus doxorubicin by continuous infusion on treated malignant lesions[a]

Patient No.	Diagnosis	Primary site	Metastasis	Recurrence	Stage or grade	Performance status, ECOG	Treatment: Doxorubicin, total mg/m^2	Treatment: RT, total cGy[b]	Response: Type	Response: Duration, wk
1	Liposarcoma	Right arm	Lung		3	2	120	Right arm: 2,000 (3,000) RUL: 2,400 (2,400)	CR	35
2	Synovial STS	Retroperitoneum	Lung, bones		3	2	240	Right hilum: 1,500 (1,500) LUQ: 2,370 (1,500)	PR	38
3	Poorly differentiated STS	Retroperitoneum		x	3	2	120	LLQ: 2,025 (1,500)	CR	188+
4	Leiomyosarcoma	Left psoas	Liver		2	1	180	Liver: 2,750	SD	27+
5	Leiomyosarcoma	Retroperitoneum		x	3	1	240	Retroperitoneum: 2,000 (2,000)	CR	204+
6	Leiomyosarcoma	Duodenum	Liver		2	0	180	Liver: 2,400 (1,500)	PR	18+
7	Fibrosarcoma	Right arm	Right axilla		3	1	240	Right axilla: 3,700 (2,800)	CR	144+
8	Fibrohistiocytoma	Paraspinal area	Ribs, retroperitoneum		2	2	240	Para-aortic mass: 3,600 (2,850)	PR	28+
9	Leiomyosarcoma	Right thigh	Lung, mediastinum		3	1	160	Upper mediastinum: 2,720	SD	NE
10	Adenocarcinoma	Stomach	Abdominal wall		IV	2	200	Abdominal wall: 1,920	SD	11
11	Adenocarcinoma	Stomach	Abdominal wall		IV	3	180	Abdominal wall: 2,340	SD	9
12	Adenocarcinoma	Colon	Omentum		D	3	120	Pelvis: 750 Liver: 1,320	Prog	—
13	Adenocarcinoma	Colon	Pelvis		D	2	120	Pelvis: 2,300	SD	5
14	Breast cancer	Breast	Breast, lung		IV	3	120	Chest wall: 1,800	Prog	—
15	Breast cancer[c]	Breast		x	IV	1	180	Breast: 3,000	SD	12
16	Breast cancer	Breast	Bones	x	IV		120	Breast: 2,000	SD	5
17	Transitional cell cancer	Bladder	Pelvis, bones, thigh		D	3	240	Left thigh: 4,860	Prog	—
18	Hepatoma	Liver		x	II	1	60	Left lobe, liver: 1,080	PR	35
19	Hepatoma	Liver		x	I	1	120	Right lobe, liver: 1,500	PR	22
20	Hepatoma	Liver	Omentum		III	1	180	Whole liver: 2,150	SD	5
21	Hepatoma	Liver	Omentum	x	II	2	140	Whole liver: 2,520	SD	12

[a] ECOG = Eastern Cooperative Oncology Group; RT = radiation therapy; RUL = right upper lobe of lung; LUQ = left upper quadrant of abdomen; LLQ = left lower quadrant of abdomen; SD = stable disease; NE = not evaluable; Prog = disease progression.

[b] Values in parentheses = total dose at time of maximum response.

[c] Inflammatory.

escalated every cycle until toxic effects developed. The number of cycles for each dose increment and the toxic effects of doxorubicin infusion are shown in table 2. Statistical considerations (*7*) have indicated that the repeated transient but severe leukopenia resulting from six consecutive cycles of doxorubicin at a dosage of 15 mg/m^2 per day was sufficient to determine that the lower dosage of 13 mg/m^2 per day was the maximum tolerable dosage.

In the second part of the phase I study, radiation therapy was given and doxorubicin was administered by continuous 5-day infusion at a dosage of 12 mg/m^2 per day. Irradiation was initially delivered in five daily fractions of 100 cGy per session to 3 patients and was repeated each time doxorubicin was administered (every 3–4 wk). The radiation dosage was escalated for each new 5-day cycle by increments of 30–50 cGy per daily session until daily conventional radiation dosages of 150 cGy per session for the trunk lesions and 200 cGy per session for the extremity lesions were reached.

Monitoring Response and Toxicity

All patients had a complete pretreatment staging workup except for laparotomy. The irradiated palpable lesions were measured before treatment and every week during and after therapy was administered. When radiological tests were required to measure the lesions, they were repeated every 6–8 weeks.

Objective responses to treatment were defined using customary criteria. Patients were monitored for cardiotoxicity. The ventricular ejection fraction was calculated initially by echocardiography, more recently by technetium-99 pool multiple-gated acquisition scan.

Complete blood counts and differential counts, liver function tests, BUN, and creatinine values were obtained at appropriate time intervals.

Pharmacokinetics of Doxorubicin

The doxorubicin levels in serum and urine were determined in 3 patients by a solid-phase RIA (*8*), using the commercially available ^{125}I-labeled doxorubicin RIA kit (Diagnostic Biochemicals, San Diego, CA), and were expressed in nanograms per milliliter. The doxorubicin levels determined by RIA represent the amounts of doxorubicin and its immediate metabolites, adriamycinol and deoxyadriamycinol aglycones, with which the anti-doxorubicin antibody has a strong cross-reaction (*8*).

RESULTS

Toxicity and Kinetics

Bone marrow suppression was the limiting factor during escalation of the doxorubicin dose administered to the first patients entered in this study (table 2). It was accompanied by mucositis, especially glossitis, and one episode of sepsis. At a doxorubicin dosage of 13 mg/m^2 per day (table 2), the blood counts had only a moderate decrease and returned to normal by days 18–21 after the start of the infusion, thus permitting a repeat of the infusion cycle every 3 weeks. However, in 7 patients, the cycles had to be delayed an additional week. Although the number of cycles administered was limited by the cardiotoxic effects of doxorubicin, continuous infusion could be safely administered up to a cumulative dose of 840 mg/m^2.

After an initial slow start, the serum level of doxorubicin administered at a dosage of 12 mg/m^2 per day (fig. 1, *upper graph*) increased within 24–30 hours almost exponentially to 60 ng/ml, where it plateaued. The disappearance of doxorubicin from plasma has been biphasic. From its disappearance curve (fig. 1), we calculated the mean half-life of doxorubicin as 20.5 hours.

The doxorubicin excretion in the urine (fig. 1, *lower graph*) had a steady rise until the continuous infusion was terminated. The cumulative urinary excretion of doxorubicin and its immediate metabolites represented 9.7% of the total dose administered during the 5-day infusion.

Toxicity of Doxorubicin Plus Radiation Therapy

The effects of escalating dosages of radiation (starting at 100 cGy/day) were assessed in 3 patients. No severe toxic effects were noted (table 2). Skin erythema and nonpitting edema were the dose-limiting factors. Subsequently, daily doses of 150 cGy were delivered to measurable lesions of the chest and abdominal cavity, and 180–200 cGy was delivered to lesions of the limbs and head and neck region in cycles of 5 days every 3 weeks, with the concurrent administration of doxorubicin. At this dose, local skin reaction at the site of administration of radiation consisted of erythema followed 4–6 weeks later by hyperpigmentation and occasionally by a slight degree of skin induration. Table 3 reveals the incidence of various toxic effects encountered during the administration of this combined modality treatment to 21 patients.

TABLE 2.—Toxicity of escalating doses of doxorubicin by continuous infusion without and with concomitant escalating doses of RT

Dose			Nadir counts				Ventricular ejection fraction[a]	
Doxorubicin (mg/m^2 per day × 5)	RT (cGy/day × 5)	No. of cycles	WBC (× $10^3/\mu l$)	Platelets (× $10^3/\mu l$)	Mucositis[b]	Dermatitis[b]	Direct	After exercise
9		3	3.6 ± 0.85	135 ± 42	0	0	55%	74%
11		9	3.1 ± 0.52	120 ± 25	0	0	ND	ND
13		28	2.2 ± 0.75	82 ± 35	+	0	53%	72%
15		6	0.55 ± 0.72	35 ± 40	++	0	52%	61%
12	100	3	2.1 ± 0.8	84 ± 27	+	+	ND	ND
12	150	5	2.2 ± 0.65	85 ± 31	+	+	ND	ND
12	200	3	2.05 ± 0.72	80 ± 28	++	+++	53%	72%

[a] Measured by pool multiple-gated acquisition scan. For direct measurement, normal values = 57% ± 8%. For measurement after exercise, normal values = 71% ± 8%. ND = not determined.

[b] + = grade I (erythema); ++ = grade II (erythema + vesicles); and +++ = grade III (superficial ulceration).

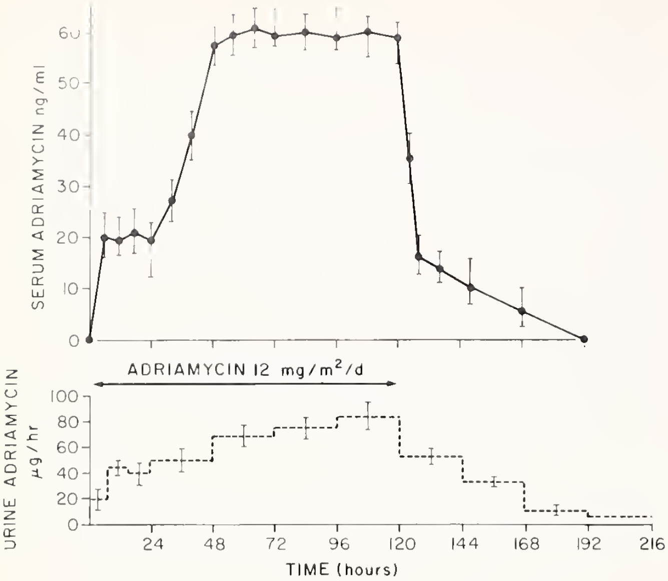


FIGURE 1.—Kinetics of doxorubicin (Adriamycin) administered by 5-day continuous infusion. Serum levels are marked on *upper graph* as mean values of 3 different determinations at same time intervals from start of infusion. *Lower graph* depicts rate of urinary excretion of doxorubicin during same period, represented by average hourly excretion calculated in each of the 3 studies.

Tumor Response

Tumor response was seen only in patients with STS and hepatoma (table 4). Of the 4 patients with hepatoma entered in this study, 2 with diffuse lesions accompanying cirrhosis of the liver had disease progression, and 2 (No. 18 and 19) who had localized metastatic lesions in the right lobe of the liver achieved PR after only two cycles of therapy, as measured by 1) decrease in the size of the lesion(s) by >70%; 2) reduction of the vertical span of the liver by 25%–30%; and 3) decrease in the serum alpha-fetoprotein level by >60%. Partial remission lasted 8½ months for patient 18; for patient 19, it was interrupted by sudden death caused by the rupture of a "berry" cerebral aneurysm. The most significant responses were noted in patients with STS. In 4 patients, CR in the treated lesions was achieved after only 1,500–2,500 cGy of radiation (table 1). In patient 3, who had undifferentiated STS, CR was achieved after failure to respond to 4,000 cGy of radiation administered alone and after two chemotherapeutic regimens; this patient developed a severe induration of the irradiated skin, but repeated biopsies did not reveal any malignant cell.

In 3 other patients, PR was achieved in the irradiated lesions after administration of a relatively low dose of radiation (1,950 ± 900 cGy).

In 2 patients (No. 4 and 6) who had leiomyosarcoma with stable disease, the total dose of radiation that could be delivered during the concurrent modality regimen was about 2,500 cGy, due to liver irradiation or previous irradiation of the same site of involvement.

In 3 patients with multiple lesions (patients 1, 2, and 8), lasting CR was achieved only in the irradiated lesions. The nonirradiated lesions have had rapid disease progression after a brief minimal response to doxorubicin.

To date, the median duration of response in the irradiated STS lesions receiving combined modality therapy is 142 ± 65 weeks for patients with CR and 28 ± 10 weeks for those with PR. The 3 patients who achieved CR of solitary recurrent lesions are still maintaining response at 204, 188, and 144 weeks. Only 1 of the patients with PR has disease progression.

DISCUSSION

These data regarding concomitant administration of radiation and doxorubicin by continuous infusion demonstrate the lack of toxicity of this combined modality therapy when administered in 5-day cycles every 3–4 weeks. They also suggest that doxorubicin given by continuous infusion has a radiation-enhancing effect in 77% of the patients with STS and in some with focal hepatocellular carcinoma, when the serum level is maintained close to 60 ng/ml. Three STS patients with multiple lesions showed a lasting response only at the lesions that were also irradiated, while 2 who were unresponsive to doxorubicin alone and to radiation alone showed marked tumor response with combined modality treatment. The 33% survival at 3 years and the 77% overall response compare favorably with the 10% survival rate previously reported in patients with stage IV STS who received radiotherapy alone (*9*); these results are similar to those of neutron beam therapy (*10*), without its incidence of major complications (32%). Importantly, the mean doses of radiation at which CR and PR were achieved (2,200 ± 590 and 1,950 ± 900 cGy, respectively) are significantly lower than those required to achieve similar results with radiation alone (>5,000 cGy). These results should be interpreted with reservation in view of two considerations: 1) the small number of patients entered in this study and 2) the fact that some patients have long-term survival after irradiation alone. It is also possible that not all histological types of STS will respond similarly to those in this study.

The selectivity of this radiation-enhancing effect of doxorubicin, which was not seen in any of the patients with adenocarcinoma of the gastrointestinal tract or breast or

TABLE 3.—Toxic effects of doxorubicin by continuous infusion with concurrent RT

Toxic effect	Incidence (%) No. of cycles ($n = 79$)	No. of patients ($n = 21$)
Skin erythema	78.1	100
Leukopenia (WBC count >1,500, <3,000/µl)	84.4	95.2
Thrombocytopenia (platelet count $>5 \times 10^4$, $<1 \times 10^5/\mu l$)	65.5	71.5
Mucositis	32.7	28.5
Infectious episodes (home treatment)	15.1	47.6
Infectious episodes (hospitalization)	3.7	14.28
Cardiac arrhythmias	3.7	14.28
Decreased ventricular ejection fraction after exercise	2.5	9.5
Cholestasis	1.2	4.75
Gastrointestinal bleeding	1.2	4.75
Pulmonary fibrosis	—	4.76
Alopecia	—	100

TABLE 4.—Response rate and duration of response in patients receiving doxorubicin infusion and concomitant RT[a]

Histology	Total No. of patients	CR		PR		Stable disease		No. of patients with disease progression
		No. of patients	No. of wk	No. of patients	No. of wk	No. of patients	No. of wk	
Adenocarcinoma of gastrointestinal tract	4	0	—	0	—	3	8 ± 3	1
Breast cancer	3	0	—	0	—	2	8 ± 4	1
Transitional cell cancer of bladder	1	0	—	—	—	0	—	1
Hepatocellular cancer	4	0	—	2 (1,290 ± 210)	28 ± 6	2	8 ± 3	0
STS	9	4 (2,206 ± 590)	142 ± 65+	3 (1,950 ± 900)	28 ± 10	2	27	0
Total		4	142 ± 65+	5	28 ± 10	9	12 ± 9	3

[a] Values in parentheses represent mean of doses of RT (in cGy) at which CR or PR was reached in each group of patients.

transitional cell carcinoma of the bladder (table 3) is difficult to explain. It could be the result of a random sampling, in view of the very small number of patients with such histology entered in this pilot study, or it may reflect a special interaction of doxorubicin with the STS and hepatoma cells, which makes them more sensitive to radiation.

These clinical data suggesting a radiosensitizing effect of doxorubicin on some malignant solid tumors confirm previous experimental data on cultured Chinese hamster ovary cells (*11*) as well as on murine, hamster (*12*), and human tumor cells (*13*). Preliminary clinical studies (*2,11,14,15*) also have indicated that doxorubicin can be safely administered in intermittent single pulses with concomitant conventional radiotherapy as well as with accelerated split-dose radiation therapy or brachytherapy. However, these data have been at variance with many other reports that indicate increased toxicity resulting from the concomitant administration of radiation and pulses of doxorubicin iv. Skin toxicity (*16*) as well as severe enteritis, esophagitis, and cardiomyopathy (*17*) have been reported.

Our data indicate that the toxicity of concomitant doxorubicin and radiation is minimal when the doxorubicin serum level does not rise to >60 ng/ml. Most remarkable was the lack of hepatic toxicity. Cardiac toxicity was also minimal. It was noted only in patients who received a total dose of 840 mg/m^2 of doxorubicin, which confirms previously reported data (*4*).

The mechanism of the radiosensitizing effect of doxorubicin is still speculative. This drug was thought (*10,11*) to be an inhibitor of mitochondrial respiratory effect, which could lead to improved oxygenation of the centrally located hypoxic cells (*18*). It is apparent that a minimum steady level of doxorubicin in the serum is necessary to maintain this effect; this is more readily achieved by continuous infusion than by intermittent iv pulses. This could also explain why the 3- to 4-week cycles are more likely to elicit the radiosensitizing effect and to increase the therapeutic index of the combined modality therapy than is a whole continuous course; the continuous course is prohibitive at a doxorubicin dosage >3 mg/m^2 per day (*19*), which generates a serum level significantly lower than the 60 ng/ml attained by the 5-day intermittent administration.

These data, if further confirmed by more extensive phase II and III clinical trials, indicate that this regimen of concurrent radiation therapy plus doxorubicin infusion has the potential to completely eradicate neoplastic cells in some recurrent as well as primary STS, with the result of lasting CR equivalent to cure, which is not attainable with the current conventional treatment.

REFERENCES

(*1*) PHILLIPS TL, FU K: Quantification of combined radiation therapy and chemotherapy effects on critical normal tissues. Cancer 37:1186–1200, 1976.

(*2*) BYFIELD JE, WATRING WG, LEMKIN SR, et al: Adriamycin: A useful adjuvant drug for combination radiation therapy. Proc Am Assoc Cancer Res ASCO 16:253, 1975.

(*3*) SHIMOYAMA M: The cytocidal action of alkylating agents and anticancer antibiotics against in vitro cultured Yoshida ascites sarcoma cells. Jpn Soc Cancer Ther 10:63–72, 1975.

(*4*) LEGHA SS, BENJAMIN RS, MACKAY B, et al: Reduction of doxorubicin cardiotoxicity by prolonged continuous intravenous infusion. Ann Intern Med 96:133–139, 1982.

(*5*) BJELETICH J, HICKMAN RO: The Hickman indwelling catheter. Am J Nurs 80:62–65, 1980.

(*6*) HASKELL CM, EILBER FR, MORTON DL: Adriamycin (NSC-123127) by arterial infusion. Cancer Chemother Rep 6:187–189, 1975.

(*7*) POCOCK SJ: The size of a clinical trial. *In* Clinical Trials (SJ Pocock, ed). New York: Wiley, 1983, pp 138–141.

(*8*) VAN VUNAKIS H, LANGONE JJ, RICEBERG LJ, et al: Radioimmunoassays for Adriamycin and daunomycin. Cancer Res 34:2546–2550, 1974.

(*9*) LINDBERG RD, MARTIN RG, ROMSDAHL MD: Surgery and post-operative radiotherapy in the treatment of soft tissue sarcomas in adults. Am J Roentgenol 123:123–129, 1975.

(*10*) CATERALL M: Observations on the reactions of normal and malignant tissues to a standard dose of neutrons. Presented at the Third Meeting on High-LET Particles in Clinical Radiotherapy, Sept 1978.

(*11*) KIMLER BF, LOEPER DB: The effect of Adriamycin and radiation on G cell survival. Int J Radiat Oncol Biol Phys 3:1297–1300, 1979.

(*12*) BYFIELD JE, LEE YC, TU L: Molecular interactions between Adriamycin and x-ray damage in mammalian tumor cells. Int J Cancer 19:186–193, 1977.

(*13*) BYFIELD JE, LYNCH M, KULHAMAN F, et al: Cellular effects of combined Adriamycin and X irradiation in human tumor cells. Int J Cancer 19:194–204, 1977.

(*14*) BLUM RH, GREENBERGER JS, WILSON RE, et al: Feasibility of combined modality therapy for localized high-grade soft tissue sarcomas in adults. Int J Radiat Oncol Biol Phys 5:1282–1285, 1979.

(*15*) Sinoff C, Falkson G, Sandison AG, et al: Combined doxorubicin and radiation therapy in malignant pleural mesothelioma. Cancer Treat Rep 66:1605–1608, 1982.

(*16*) Cassidy JR: Radiation-Adriamycin interactions: Preliminary clinical observations. Cancer 36:946–950, 1975.

(*17*) Greco AA, Brereton HD, Kent H, et al: Adriamycin and enhanced radiation reaction in normal esophagus and skin. Ann Intern Med 85:294–298, 1976.

(*18*) Durand RE: Adriamycin: A possible indirect radiosensitizer of hypoxic tumor cells. Radiology 119:217–222, 1976.

(*19*) Lokich J, Bothe A, Zipoli T, et al: Constant infusion schedule for Adriamycin: A phase I–II clinical trial of a 30 day schedule by ambulatory pump delivery system. J Clin Oncol 1:24–28, 1983.

Treatment of High-risk Sarcomas in Children and Young Adults: Analysis of Local Control Using Intensive Combined Modality Therapy

Timothy J. Kinsella,[1],* James S. Miser,[1] Timothy J. Triche,[1] Klara Horvath,[2] and Eli Glatstein[1]

ABSTRACT—**Although combination chemotherapy and local irradiation are quite effective treatment for some children and young adults with small round cell sarcomas, high-risk patient groups, including patients with localized disease of the trunk and proximal extremity and those who present with metastases, continue to fare poorly with standard combined modality therapy. In an attempt to improve the local and systemic response of these tumors, an intensive treatment protocol was designed that integrates five cycles of chemotherapy with vincristine, doxorubicin, and cyclophosphamide (VADRIAC) plus radiation therapy to the primary tumor (55–60 Gy), bone, and soft tissue metastases (45–50 Gy). Patients achieving complete response to this induction therapy receive intensification treatment with total body irradiation (8.0 Gy), a cycle of VADRIAC, and autologous bone marrow transplantation. All treatment is completed within 6–7 months. From January 1983 to February 1986, 76 consecutive, previously untreated patients were entered in this study; 75 patients are evaluable. Twenty-five patients were diagnosed with rhabdomyosarcoma, 23 with Ewing's sarcoma, 15 with primitive neuroepithelioma, 12 with primitive sarcoma, and 1 patient with metastatic neuroblastoma. Forty-three patients (57%) had metastases at presentation. Overall, 68 of 75 patients (91%) achieved complete response. Fifty-eight of 61 patients with measurable soft tissue masses at the primary site had ≥50% tumor reduction with two cycles of chemotherapy prior to local irradiation. Seven patients failed to have complete response at the primary site following five cycles of chemotherapy and local external beam irradiation, although 3 were subsequently rendered locally disease free by intraoperative radiotherapy (2 patients) or surgery (1 patient). With a median follow-up of 20 months from diagnosis, 66 patients (87%) have maintained local control. Actuarial overall survival and disease-free survival at 24 months are 68% and 48%, respectively. Seven patients have developed significant treatment-related local complications, including bone fracture requiring amputation (1 patient); function-limiting contractures (4 patients); and vaginal stenosis (2 patients). With follow-up ≤3 years, we conclude that this intensive combined modality approach results in excellent local control with an acceptable risk of significant local complications.—NCI Monogr 6:291–296, 1988.**

Over the last 10–15 years, there has been an improvement in overall survival of children and young adults with small round cell sarcomas through the use of combination chemotherapy with local irradiation and/or surgery (*1–7*). However, many patients with disease arising in the trunk and proximal extremity and most patients who present with metastases continue to have a poor prognosis when standard therapy is used (*4,8–14*). Analyses of data by the IESS, the IRS, and single institutions such as the NCI define these high-risk patient groups who continue to have unacceptably high rates of local and systemic failures (*4,8–14*). For example, the IESS recently published their results in patients with localized pelvic disease (*15*). Of 62 patients, 27% failed locally and 61% failed systemically. With a median follow-up of 2.5 years, only 30% of the patients remain disease free. Similar results are found for patients with stage III rhabdomyosarcoma arising in the chest wall, retroperitoneum, perineum, and extremity (*4,11,12*). Only 50%–60% of patients who present with metastases have achieved CR in previous IESS and IRS protocols, and a majority of the responders relapsed within 1–2 years (*4,10,12*). Additionally, peripheral neuroepithelioma is a recently recognized pathological diagnosis within the differential of small round cell tumors and appears to have a similarly poor prognosis based on the limited clinicopathological series reported in the literature (*16–20*).

In an attempt to improve the local and systemic response in these high-risk patient groups, we designed an intensive combined modality protocol. Five cycles of VADRIAC are given at 3-week intervals in combination with high-dose radiation therapy to the primary tumor (55–60 Gy) and sites of gross metastatic disease, except for pulmonary metastases (45–50 Gy). No surgical treatment is planned. In patients achieving CR to this induction therapy, intensification therapy is given with TBI (8 Gy), a cycle of high-dose chemotherapy, and autologous bone marrow transplantation. This report analyzes local tumor response and complications in 76 consecutive patients entered in this study over a 3-year period.

MATERIALS AND METHODS

Patient Population

Previously untreated children and young adults with metastatic sarcoma or unresectable localized sarcoma at

---

ABBREVIATIONS: IESS = Intergroup Ewing's Sarcoma Study; IRS = Intergroup Rhabdomyosarcoma Study; NCI = National Cancer Institute; CR = complete response; VADRIAC = vincristine, doxorubicin, and cyclophosphamide; TBI = total body irradiation; CT = computerized tomography; IORT = intraoperative radiotherapy; PR = partial response; OS = overall survival; DFS = disease-free survival.

[1] Radiation Oncology Branch (*T. J. Kinsella* and *E. Glatstein*) and Pediatric Branch (*J. S. Miser*), Clinical Oncology Program, Division of Cancer Treatment, and Laboratory of Pathology, Division of Cancer Biology and Diagnosis (*T. J. Triche*), National Cancer Institute, National Institutes of Health, Bethesda, MD.

[2] Department of Diagnostic Radiology, Clinical Center, National Institutes of Health, Bethesda, MD.

* *Reprint requests:* Timothy J. Kinsella, M.D., Department of Human Oncology, K4/312, Wisconsin Clinical Cancer Center, 6000 Highland Ave., Madison, WI 53792.

high-risk sites were eligible for this protocol. These sarcomas and sites included Ewing's sarcoma arising in the pelvis, humerus, femur, or rib; alveolar rhabdomyosarcoma (stage III) arising in the extremity, chest wall, retroperitoneum, or perineum; and peripheral neuroepithelioma and primitive (unclassified) round cell sarcomas occurring in soft tissue or bone of the trunk and proximal extremity. Patients with metastases at presentation were considered eligible for study regardless of the site of the primary tumor. Informed consent outlining the potential risks and benefits of this investigational study was obtained from the patient or guardian prior to entry.

In addition to a complete history and physical examination, the disease was staged prior to treatment after performance of chest x-ray, x-ray of the primary site, CT of the primary tumor and chest, bone scan, and routine blood tests and after bilateral posterior iliac crest bone marrow biopsies and aspirates were obtained. If the primary tumor arose in the iliac bone, a positive ipsilateral bone marrow biopsy or aspirate was not considered to indicate a metastatic site. Re-evaluation of all sites of disease was performed after two cycles of chemotherapy (6 wk); after five cycles of chemotherapy and local irradiation (16–18 wk); following completion of all therapy (3, 6, and 12 mo); and every 6 months thereafter. Patients were also evaluated for treatment-related complications at these follow-up times.

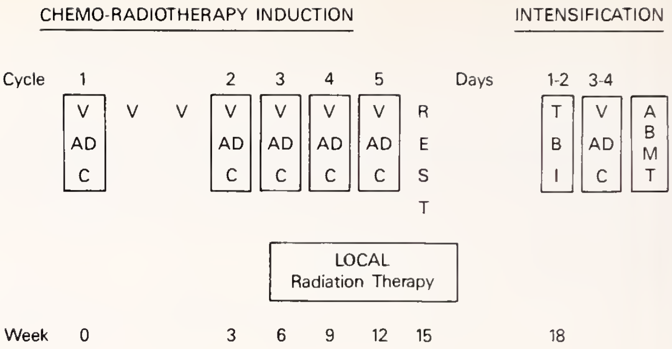
Pathology

All pathology specimens were evaluated by a single pathologist (T. J. Triche). Cases were classified as either Ewing's sarcoma, rhabdomyosarcoma, peripheral neuroepithelioma, or primitive (unclassified) round cell sarcoma. The minimum pathological examination necessary for diagnosis included adequate light microscopy and immunocytochemistry using neuron-specific enolase, creatine kinase MM isoenzyme, myoglobin, and skeletal muscle myosin. Material for electron microscopy was obtained when possible. The specific pathological criteria used to diagnose these tumors are outlined in detail in previous publications (*21,22*).

Therapy Protocol

This intensive combined modality protocol is outlined in figure 1. Patients received five cycles of VADRIAC every 3 weeks with the doses indicated in figure 1. Two cycles of chemotherapy were given prior to local irradiation. Radiation therapy was used during the first 6 weeks only when there were symptoms of direct neurological damage by tumor invasion or compression of the peripheral or central nervous system. Typically, radiation therapy was begun at week 7 and continued during the third, fourth, and possibly fifth cycles of chemotherapy. When significant skin or mucosal toxicity resulted from concomitant drug and radiation therapy in the fourth cycle, the fifth cycle was delayed (usually <2 wk) to avoid a second break in radiation therapy.

The primary tumor and metastases, excluding multiple lung nodules, were irradiated simultaneously using conventional fractionation (1.8–2.0 Gy) to a total dose of 55–60 Gy to the primary tumor and 45–50 Gy to metastases. Isocentric treatment using contrast-enhanced, CT-assisted treatment planning was used with care to include the soft tissue extension around the primary tumor and metastases. The initial primary tumor volume was treated to a total dose of 45–50 Gy, followed by a 10-Gy boost to the residual soft tissue/bone abnormality defined by CT scan and bone scan. Bone metastases were treated with fields limited to abnormalities shown on the bone scan and plain radiograph and any soft tissue extension with a 2-cm margin.



DOSES:
Vincristine (V) = 2 mg/m^2 (max = 2.0 mg)
Adriamycin (AD)
Week 0 and 3 = 45 mg/m^2/day × 2 days
Week 6, 9, and 12 = 30 mg/m^2/day × 2 days
Week 18 = 35 mg/m^2/day × 2 days
Cyclophosphamide (C)
Week 0, 3, 6, 9, and 12 = 900 mg/m^2/day × 2 days
Week 18 = 1200 mg/m^2/day × 2 days
Total Body Irradiation (TBI) = 4.0 Gy/day × 2 days
Autologous Bone Marrow Transplantation (ABMT)

FIGURE 1.—Outline of combined modality treatment for high-risk sarcomas in children and young adults.

In patients with localized pelvic primary tumors who had a persistent soft tissue mass following completion of five cycles of chemotherapy and local external beam irradiation, repeat percutaneous biopsy of the residual mass was performed. If the biopsy was positive, patients were offered IORT in an attempt to render them locally disease free. These patients had exploratory surgery; the residual soft tissue mass was exposed; and an IORT dose of 20 Gy was delivered using appropriate electron energies. There was no attempt at surgical resection. Our technique of IORT has been published in detail (*23*).

Only patients achieving CR to the initial five cycles of chemotherapy and irradiation were eligible to receive intensification therapy (fig. 1). Patients received 4.0-Gy fractions of TBI on days 1 and 2; TBI was delivered using 15-MeV photons with a dose rate of 0.15 Gy/minute. Chemotherapy with VADRIAC was given on days 3 and 4, and autologous bone marrow transplantation was performed on day 5 or 6. Patients remained hospitalized following intensification therapy until the absolute granulocyte count was >500 cells/mm^3.

Bone Marrow Storage and Transplantation

Following at least two cycles of chemotherapy and usually prior to radiation therapy, the bone marrow was harvested by standard aspiration under general anesthesia.

Patients with bone marrow metastases at presentation were eligible for harvest only after they had received five cycles of VADRIAC and after the bone marrow was clear of tumor for at least 6 weeks. Bone marrow was aspirated from the posterior iliac crests and, when necessary, from the anterior iliac crests and sternum. Mononuclear cells were separated on Ficoll-Paque (Pharmacia) and cryopreserved in 10% dimethylsulfoximide and 20% autologous plasma in TC-199 medium using a programmable freezer. A minimum of 5×10^8 cells/kg of body weight were stored in the liquid phase of a liquid-nitrogen freezer. Following intensification therapy (day 5 or 6), the bone marrow was thawed at the bedside and reinfused through a central iv line.

TABLE 1.—Histology and extent of disease at diagnosis

Histology	No. of patients Local disease	Metastasis	Total
Rhabdomyosarcoma[a]	9	16	25
Ewing's sarcoma	10	13	23
Peripheral neuroepithelioma	7	8	15
Primitive sarcoma	7	5	12
Neuroblastoma	0	1	1
Total	33	43	76

[a] Includes alveolar histology, 19 patients; embryonal histology, 5 (all metastatic); and other histology, 1 patient (metastatic).

Response Criteria

A CR was defined as complete resolution of all clinical, radiographic, and pathological evidence of malignancy. Bone scan abnormalities had to show marked improvement, with complete resolution of any contiguous soft tissue mass, although some bone scans remained slightly abnormal for several months as the diseased bone remodeled. Similarly, involved bones had to show evidence of healing by CT scan and plain radiographs. To avoid fracture, we did not routinely require a biopsy at the site of a primary bone tumor, particularly in weight-bearing bones. However, following five cycles of chemotherapy and radiation therapy, patients with a residual soft tissue mass, residual pulmonary nodules, or initial bone marrow metastases had another biopsy taken. It should be re-emphasized that only patients achieving CR were eligible for intensification therapy.

A PR was defined as ≥50% reduction in the soft tissue mass(es). The products of the two greatest perpendicular diameters of the soft tissue mass(es) were used to approximate the tumor volume. Complete clinical restaging was performed after two cycles of chemotherapy (6 wk) and after five cycles of chemotherapy and radiation therapy (16–18 wk) to assess response. Following completion of all therapy, patients were fully evaluated at 3, 6, and 12 months and every 6 months thereafter.

Statistical Considerations

Complete follow-up data were available on all patients entered in this study. The OS time was calculated from the start of therapy to the date of the most recent follow-up or to the time of death, using the Kaplan-Meier method *(24)*. The DFS was measured from the time when CR was achieved either to the last follow-up or to the time when disease recurred locally or at distant sites. Local DFS was measured from the time when local CR was achieved either to the last follow-up or to the time when disease recurred locally.

RESULTS

Patient Population

From January 1983 to February 1986, 76 patients with high-risk small round cell tumors were entered in this trial. The pertinent histological features of the patient population are outlined in table 1. There were 52 males and 24 females with a median age of 18 years (range, 2–35). Forty-three patients (57%) presented with metastases, often with multiple sites of metastatic disease. The sites of metastases were bone marrow (22 sites), lung (22), bone (15), and lymph node (9). The patient population included 25 patients with rhabdomyosarcoma, 23 with Ewing's sarcoma, 15 with peripheral neuroepithelioma, 12 with primitive (undifferentiated) sarcomas of bone and soft tissue, and 1 patient with neuroblastoma.

Local Response

Of the 76 patients entered in the study, 1 patient withdrew after two cycles and is not evaluable for response. Sixty-eight of 75 evaluable patients (91%) were judged to have CR to five cycles of VADRIAC chemotherapy and local external beam irradiation. Local control of the primary tumor has been maintained in 66 patients (87%), with a median follow-up of 20 months since the start of treatment and 13 months since local control was established (fig. 2). Of the 7 patients not achieving local CR, only 1 patient was judged to have progressive disease. This patient presented with a primitive sarcoma of the biliary tree and showed disease progression along biliary radicals throughout both the right and left liver lobes. The other 6 patients achieved PR of the local tumor mass. Three of these patients had PR and positive tumor biopsy of a residual soft tissue mass, and further local treatment was attempted to render them locally disease free and eligible for intensification therapy. Two patients with Ewing's sarcoma arising in the pelvis received an additional 20 Gy of electron beam IORT without any tumor resection. Both patients failed systemically following intensification therapy, with continued local control during follow-up of 15 and 18 months after completion of treatment. A third patient had a persistent alveolar rhabdomyosarcoma of the forearm, which was grossly excised with a limb-sparing procedure, followed by an additional postoperative boost of 14 Gy using 2-Gy fractions of external electron therapy. Again, local control has been maintained for ≤15 months. Since local control was not achieved with the planned therapy of five cycles of chemotherapy and 55–60 Gy of external beam irradiation, these 3 patients were considered as treatment failures in the analysis of figure 2, even though ultimate local control was established by further local treatment and was maintained. Another patient, who had metastatic neuroblastoma, underwent resection of a persistent retroperitoneal mass without additional radiation therapy but failed locally shortly following resection.

To assess the relative impact of induction chemotherapy and local irradiation on local control of the primary tumor, we attempted to measure response of soft tissue tumor (both primary soft tissue tumors and soft tissue extension of primary bone tumors) by CT scan following two cycles of chemotherapy (week 6 of protocol) and following external beam irradiation and an additional three cycles of chemotherapy (weeks 16–18 of protocol). Of 61 patients with measurable soft tissue masses defined by CT scan prior to treatment, 58 (95%) had ≥50% reduction (PR) following the initial two cycles of chemotherapy. A CR was documented in 55 of these patients following five cycles of chemotherapy and local external beam irradiation. A typical response of the soft tissue tumor mass (usually massive; i.e., >10 cm in diameter) is shown in figure 3. While it was not possible to quantitate tumor response in metastatic bone and soft tissue sites, there has been no instance of tumor recurrence in a previously irradiated metastatic site. Additionally, we have been able to assess the ability of chemotherapy to treat bone marrow metastases. Twenty of 22 patients (91%) had CR in bone marrow as defined by three separate negative bone marrow aspirates and biopsies following five cycles of chemotherapy.

Overall Response

With a median follow-up of 20 months since initiation of therapy, 52 of 76 patients remain alive. Twenty patients died of disease and 4 died of toxicity following intensification therapy (5% treatment-related mortality). The actuarial OS and DFS rates at 24 months are 68% and 48%, respectively (fig. 4). A more complete analysis of this protocol with respect to prognostic variables, treatment results, and systemic toxicity will be presented elsewhere.

Local Complications

Most patients developed mild to moderate acute complications within the radiation field, usually requiring a 10- to 14-day treatment break. As stated previously, if patients required a significant radiation treatment break following the fourth cycle of chemotherapy (usually at 30–45 Gy of the planned radiation dose), the fifth cycle of chemotherapy was delayed until all local radiation therapy was completed. Signs and symptoms of acute local toxicity varied with the location of the primary tumor and metastatic sites. However, all 75 evaluable patients completed the planned local irradiation.

To date, 7 patients (9%) developed significant late (>6 mo) local complications, which were judged to be treatment related. Among 24 extremity primary sarcomas, only one bone fracture has occurred. A displaced, noncomminuted fracture of the femur resulted following minor trauma, and

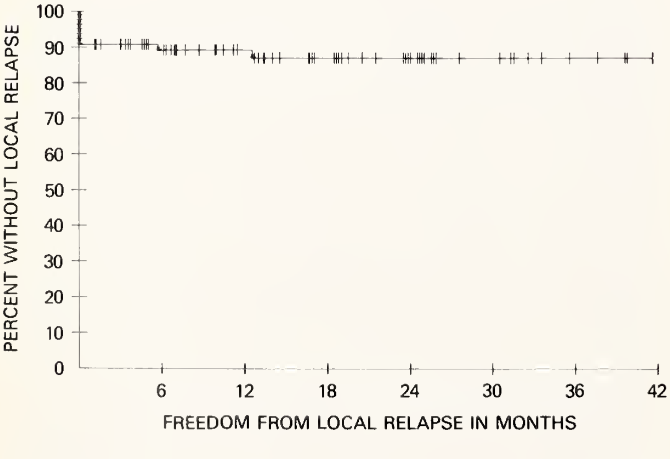


Figure 2.—Analysis of local DFS. Median follow-up from achievement of local control of primary tumor is 13 mo; from start of therapy, it is 20 mo.

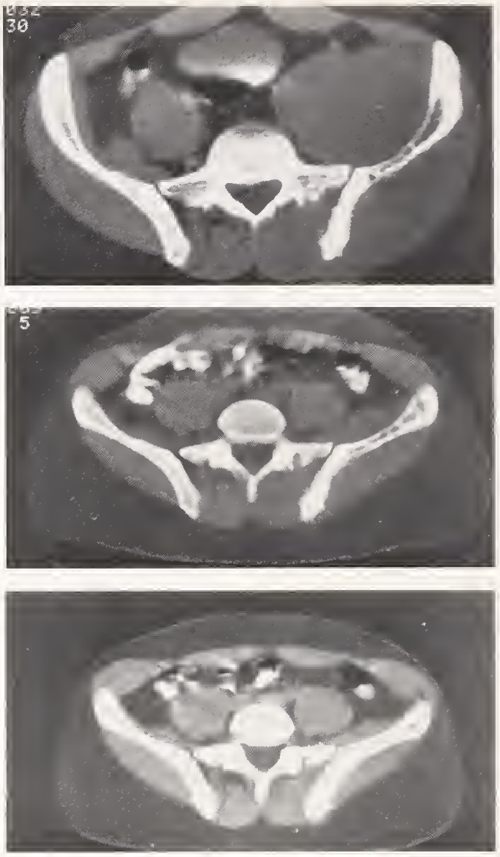


Figure 3.—Typical response of soft tissue tumor mass following 2 cycles of chemotherapy (*middle*) and following completion of intensification therapy (*bottom*), compared to pretreatment mass (*top*).

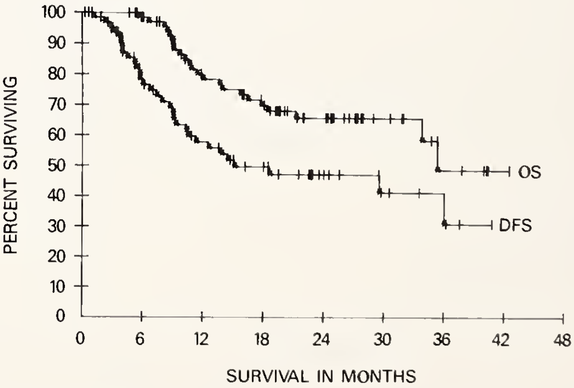


Figure 4.—Actuarial OS and DFS curves for patients with high-risk sarcoma, using Kaplan-Meier method.

a hip disarticulation developed when postfixation complications occurred. In 4 patients, joint contractures developed secondary to progressive soft tissue fibrosis. Two of the 24 patients with extremity primary tumors have knee contractures, which have resulted in persistent limp with walking, although without pain. Two of the 24 patients with pelvic primary tumors have flexion contractures of the hip, probably resulting from the additional use of IORT (20 Gy) to a tumor volume that included the acetabulum. In one of these patients, osteomyelitis developed in the ilium following a post-treatment biopsy at another institution, and this infection further complicated hip function. Finally, 2 additional patients with pelvic primary tumor developed vaginal stenosis, which was partially corrected by regular vaginal dilation.

DISCUSSION

Because of the unacceptably high rates of local and systemic failure in these high-risk patients with small round cell sarcoma (*4,8–15*), we have investigated the use of short but very intensive induction therapy with VADRIAC chemotherapy and high-dose local irradiation to the primary tumor and metastatic sites. Additionally, we have replaced the traditional use of maintenance chemotherapy in patients with CR with a consolidative regimen utilizing high-dose chemotherapy, high-dose TBI, and autologous bone marrow transplantation. In light of the risk of radiation pneumonitis with TBI, multiple pulmonary nodules did not receive high-dose local irradiation. At this time, we conclude that the induction therapy is quite effective; the CR rate is >90%. With a median follow-up of 20 months, a time when many high-risk patients have died on previous protocols (*4,8–15*), 68% of the patients are alive and 48% are disease free. Longer follow-up is necessary, however, to assess more accurately the impact of this induction and consolidation therapy approach.

In the analysis of local control, we have documented that most patients will have a significant response (≥50% tumor reduction) to chemotherapy alone (two cycles) and that >85% will remain locally disease free following such combined modality therapy. The predominant pattern of failure remains systemic in previously uninvolved sites. Given that >50% of our high-risk patients presented with metastases and 50% failed systemically, one must conclude that these pediatric and young adult sarcomas are truly systemic diseases. Thus, local treatment may be less important than systemic treatment and should not compromise the delivery of effective systemic treatment.

Our philosophy in designing induction therapy was to assess initial response to chemotherapy (two cycles) and then to integrate local irradiation to the primary tumor and gross metastatic sites during the subsequent three cycles of chemotherapy. With close communication between the pediatric and radiation oncologists, 75 of 76 patients completed the planned induction therapy with acceptable toxicity and no treatment-related mortality. One might question whether local treatment is necessary in view of the often significant response of the primary tumor to two cycles of chemotherapy (fig. 3). This question was not addressed in the protocol since we believed that gross primary tumor (typically very bulky; i.e., >10 cm in diameter) and overt metastatic disease are unlikely to be sterilized permanently with chemotherapy alone. Certainly, previous chemotherapy trials in metastatic patients by the IESS, IRS, and NCI have shown that only 50%–60% have CR and a majority of these patients relapse (*4,8–15*).

Recently, it has been suggested that radical surgery with either amputation or wide excision should be considered as an alternative to radiation therapy in the local management of small round cell sarcomas, particularly Ewing's sarcoma (*25,26*). The precise definition of such surgery and the resultant functional outcome have not been presented, however. Our group and others have shown that local irradiation and systemic chemotherapy are very effective together in maintaining long-term local control and function in distal extremity (low-risk) tumors (*27–31*). In high-risk patients with bulky proximal extremity or truncal primary tumors, wide excision is simply not possible in many patients at presentation. Moreover, in many patients, it may delay systemic therapy, which is clearly critical in a curative strategy.

Preoperative chemotherapy (three to five cycles) has been used to reduce tumor bulk and allow for wide excision of selected extremity, rib, and pelvic Ewing's sarcoma at other institutions (*26,32*). Moderate-dose radiation therapy (35–40 Gy) was used postoperatively in patients with gross or microscopic disease documented at surgery. Combination of the results of this approach in selected patients from these two institutions shows that approximately 60% (29 of 50 patients) still required postoperative irradiation. No objective analyses of local control and of functional results are available from these trials at this time to compare to our results in this larger, nonselected series. Certainly, in high-risk patients who present with metastases, a major ablative surgical procedure (i.e., amputation, hemipelvectomy, or chest wall resection) would be difficult to justify in light of 1) the systemic nature of the disease; 2) the major functional compromise of this type of surgery; and 3) our excellent local control data with follow-up ≤3 years after intensive combined modality therapy.

REFERENCES

(*1*) HEYN RM, HOLLAND R, NEWTON WA, et al: The role of combined chemotherapy in the treatment of rhabdomyosarcoma in children. Cancer 34:2128–2142, 1974.

(*2*) GRAHAM-POLE J: Ewing's sarcoma: Treatment with high dose radiation and adjuvant chemotherapy. Med Pediatr Oncol 7:1–8, 1979.

(*3*) BACCI G, PICCI P, GITEUS S, et al: The treatment of localized Ewing's sarcoma. Cancer 49:1561–1570, 1982.

(*4*) MAURER HM: The Intergroup Rhabdomyosarcoma Study: Update, November 1978. Natl Cancer Inst Monogr 56:61–68, 1981.

(*5*) NESBIT ME JR, PEREZ CA, TEFFT M, et al: Multimodal therapy for the management of primary, nonmetastatic Ewing's sarcoma of bone: An Intergroup Study. Natl Cancer Inst Monogr 56:255–262, 1981.

(*6*) ROSEN G, CAPARROS B, NIRENBERG A, et al: Ewing's sarcoma: Ten year experience with adjuvant chemotherapy. Cancer 47:2204–2213, 1981.

(*7*) HAYES FA, THOMPSON EI, HUSAU HO, et al: The response of Ewing's sarcoma to sequential cyclophosphamide and Adriamycin induction therapy. J Clin Oncol 1:45–51, 1983.

(*8*) GEHAN EA, NESBIT ME JR , BURGERT EO JR, et al: Prognostic factors in children with Ewing's sarcoma. Natl Cancer Inst Monogr 56:273–278, 1981.

(*9*) Glaubiger DL, Makuch R, Schwarz J, et al: Determination of prognostic factors and their influence on therapeutic results in patients with Ewing's sarcoma. Cancer 45:2213–2219, 1980.

(*10*) Vietti TJ, Gehan EA, Nesbit ME Jr, et al: Multimodal therapy in metastatic Ewing's sarcoma: An Intergroup Study. Natl Cancer Inst Monogr 56:279–284, 1981.

(*11*) Raney RB, Gehan EA, Maurer HM, et al: Evaluation of intensified chemotherapy in children with advanced rhabdomyosarcoma (clinical groups III and IV). Cancer Clin Trials 2:19–28, 1979.

(*12*) Maurer HM, Foulkes M, Gehan EA, et al: Intergroup Rhabdomyosarcoma Study II. Preliminary report. Proc ASCO 2:70, 1983.

(*13*) Kinsella TJ, Glaubiger D, Diesseroth A, et al: Intensive combined modality therapy including low-dose TBI in high-risk Ewing's sarcoma patients. Int J Radiat Oncol Biol Phys 9:1955–1960, 1983.

(*14*) Ruymann RB, Newton WA, Ragab A, et al: Bone marrow metastases at diagnosis in children and adolescents with rhabdomyosarcoma: A report from the Intergroup Rhabdomyosarcoma Study. Cancer 53:368–373, 1984.

(*15*) Evans R, Nesbit M, Askin F, et al: Local recurrence rate and sites of metastases, and time to relapse as a function of treatment regimen, size of primary and surgical history in 62 patients presenting with non-metastatic Ewing's sarcoma of the pelvic bones. Int J Radiat Oncol Biol Phys 11:129–136, 1985.

(*16*) Askin FB, Rosai J, Sibley RK, et al: Malignant small cell tumor of the thoracopulmonary region in childhood. Cancer 43:2438–2451, 1979.

(*17*) Harper PG, Pringle J, Souhami RL: Neuroepithelioma—A rare malignant peripheral nerve tumor of primitive origin. Cancer 48:2282–2287, 1981.

(*18*) Hashimoto H, Enjoji M, Nakajima T, et al: Malignant neuroepithelioma (peripheral neuroblastoma): A clinicopathologic study of 15 cases. Am J Surg Pathol 7:309–318, 1983.

(*19*) Gonzalez-Crussi F, Wolfson SL, Misugi K, et al: Peripheral neuroectodermal tumors of the chest wall. Cancer 54:2519–2527, 1984.

(*20*) Voss BL, Pysher TJ, Humphrey GB: Peripheral neuroepithelioma in childhood. Cancer 54:3059–3064, 1984.

(*21*) Triche TJ, Askin LB: Neuroblastoma and the differential diagnosis of small, round, blue-cell tumors. Hum Pathol 44:569–595, 1983.

(*22*) Tsokos M, Linnoile RJ, Triche TJ: Neuron-specific enolase in the diagnosis of neuroblastoma and other small, round cell tumors in children. Hum Pathol 15:575–584, 1984.

(*23*) Fraass BA, Miller RW, Kinsella TJ, et al: Intraoperative radiation therapy at the National Cancer Institute: Technical innovations and dosimetry. Int J Radiat Oncol Biol Phys 11:1299–1312, 1985.

(*24*) Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481, 1958.

(*25*) Pritchard DJ: Indications for surgical treatment of localized Ewing's sarcoma of bone. Clin Orthop 153:39–43, 1980.

(*26*) Marcove RC, Rosen G: Radical en bloc excision of Ewing's sarcoma. Clin Orthop 153:86–91, 1980.

(*27*) Tepper J, Glaubiger D, Lichter A, et al: Local control of Ewing's sarcoma of bone with radiotherapy and combination chemotherapy. Cancer 46:1969–1973, 1980.

(*28*) Jentzsch K, Binder H, Cramer H, et al: Leg function after radiotherapy for Ewing's sarcoma. Cancer 47:1267–1278, 1981.

(*29*) Kliman M, Harwood AR, Jenkin RD, et al: Radical radiotherapy as primary treatment for Ewing's sarcoma distal to the elbow and knee. Clin Orthop 165:233–238, 1982.

(*30*) Kinsella TJ, Loeffler JS, Fraass BA, et al: Extremity preservation by combined modality therapy in sarcomas of the hand and foot: An analysis of local control disease-free survival and functional result. Int J Radiat Oncol Biol Phys 9:1115–1119, 1983.

(*31*) Kinsella TJ, Lichter AS, Miser J, et al: Local treatment of Ewing's sarcoma: Radiation therapy versus surgery. Cancer Treat Rep 68:695–701, 1984.

(*32*) Hayes FA, Thompson EI, Hustu HO, et al: The response of Ewing's sarcoma to sequential cyclophosphamide and Adriamycin induction therapy. J Clin Oncol 1:45–51, 1983.

Integration of Full-dose Adjuvant Chemotherapy With Definitive Radiotherapy for Primary Breast Cancer: Four-year Update[1]

John H. Glick,* Barbara L. Fowble, Daniel G. Haller, Ernest F. Rosato, Julius A. Mackie, Clare Weiler, Donna J. Glover, Kevin R. Fox, Shelley Hurwitz, and Robert L. Goodman[1,2]

ABSTRACT—Controversy exists over the effect of definitive radiotherapy on the ability to administer full doses of adjuvant chemotherapy in primary breast cancer. Ninety-six consecutive women with clinical stage I and II breast cancer were treated with radiotherapy plus chemotherapy. Three combinations of drugs were used: cyclophosphamide and 5-fluorouracil (CF); cyclophosphamide, methotrexate, and 5-fluorouracil (CMF); or cyclophosphamide, methotrexate, 5-fluorouracil, and prednisone (CMFP). Chemotherapy consisted of two cycles of CF (cyclophosphamide at a dosage of 100 mg/m^2 orally on days 1-14 + 5-fluorouracil at 600 mg/m^2 iv on days 1 and 8) during concurrent radiotherapy, followed by six cycles of CMFP (same CF dosages + methotrexate at 40 mg/m^2 iv on days 1 and 8 + prednisone at 40 mg/m^2 orally on days 1-14). The study included 63 premenopausal and 33 postmenopausal patients; 72 had 1-3 positive nodes, 15 had ≥4 positive nodes, and 9 had negative nodes and negative estrogen receptors. The mean CF doses delivered during concurrent radiotherapy were 95% of the optimal doses, and the mean CMF doses administered during the six cycles after radiotherapy were 89%. The CMF was delivered at level I (≥85% of optimal doses) to 73% of the patients. With a median follow-up of 36 months, 16 relapses have been observed. Two of these patients had treatment failure only in the breast or axilla and are disease free after mastectomy. Of the 72 patients with 1-3 positive nodes, 10 relapsed in distant sites, while 4 of 15 patients with ≥4 positive nodes have had distant failure. None of the 9 patients with negative nodes and negative estrogen receptors have had failure. Actuarial relapse-free survival at 4 years is 77% for all patients, 78% for those with 1-3 positive nodes, and 58% for those with ≥4 positive nodes. Actuarial overall survival at 4 years is 93% for all patients, 97% for those with 1-3 positive nodes, and 74% for those with ≥4 positive nodes. We conclude that full doses of adjuvant chemotherapy can be safely and effectively administered on schedule to patients undergoing definitive radiotherapy for primary breast cancer.—NCI Monogr 6:297-301, 1988.

Conservative surgery plus definitive radiotherapy has become an accepted alternative to total mastectomy for early carcinoma of the breast (*1,2*). The goal of primary irradiation is enhanced cosmesis with breast preservation but without sacrificing an equivalent opportunity for cure. With this approach, locoregional tumor control is equivalent to that with mastectomy (*2*). However, ultimate cure depends on the appearance of metastatic disease. The systemic nature of breast cancer even at the time of primary diagnosis and local therapy is well recognized. The high correlation between the presence of positive axillary nodes at the time of mastectomy and an increased risk of relapse has led to the development of adjuvant chemotherapy trials designed to reduce the relapse rate and improve overall survival (*3-5*). The recent NIH Consensus Development Panel on Adjuvant Chemotherapy and Endocrine Therapy for Breast Cancer concluded that adjuvant chemotherapy and hormonal therapy after mastectomy have resulted in significant advances in the prognosis of many subsets of primary breast cancer but that optimal therapy has not yet been defined for all patients (*6*).

Improvements in relapse-free survival and overall survival would also be expected in patients treated with conservative surgery, definitive radiotherapy, and adjuvant chemotherapy. It has been suggested that the doses and rates of drug administration are critical variables in determining the prognosis of patients receiving adjuvant chemotherapy after mastectomy (*7,8*). However, the effect of primary irradiation on drug doses and the schedule of adjuvant chemotherapy is controversial. This report presents the updated results of the University of Pennsylvania experience in 96 consecutive patients treated with definitive irradiation and adjuvant chemotherapy for primary breast cancer.

MATERIALS AND METHODS

Between January 1978 and December 1985, 96 consecutive patients were entered in a prospective trial of definitive radiotherapy and adjuvant chemotherapy for primary breast cancer. Eligibility criteria included premenopausal or postmenopausal status in women ≤75 years of age who were candidates for primary radiotherapy with clinical stages I and II (American Joint Committee on Cancer); excisional biopsy of the breast primary tumor and limited axillary lymph node dissection; absence of distant metastases; and normal hematologic, renal, and hepatic functions. Initially, only patients with one or more histologically positive axillary nodes were included. However, since

---

ABBREVIATIONS: NIH = National Institutes of Health; CF = cyclophosphamide and 5-fluorouracil; CMF = cyclophosphamide, methotrexate, and 5-fluorouracil; CMFP = cyclophosphamide, methotrexate, 5-fluorouracil, and prednisone.

---

[1] Supported in part by Public Health Service grant CA-16520 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services. J. H. Glick is the recipient of a Faculty Research Award from the American Cancer Society.

[2] Hematology-Oncology Section (*J. H. Glick, D. G. Haller, C. Weiler, K. R. Fox,* and *D. J. Glover*), Department of Radiation Therapy (*B. L. Fowble* and *R. L. Goodman*), Department of Surgery (*E. F. Rosato* and *J. A. Mackie*), and Biostatistical Unit (*S. Hurwitz*), University of Pennsylvania Cancer Center and School of Medicine, Philadelphia.

* *Reprint requests:* John H. Glick, M.D., Hematology-Oncology Section, University of Pennsylvania Cancer Center, Hospital of the University of Pennsylvania, 3400 Spruce St., Philadelphia, PA 19104.

March 1983, patients with negative nodes were entered if the estrogen receptors on the primary tumor were negative.

Surgery consisted of lumpectomy or excision of all gross tumor. While a margin of histologically normal tissue was not required, the majority of patients underwent resection of the primary tumor with a rim of adjacent normal breast tissue. Patients had an axillary lymph node dissection, which corresponds to removal of the level I or level I and II lymph nodes.

Radiotherapy began within 10–21 days of axillary surgery, provided that the incision was adequately healed. All patients were treated with opposed tangential fields to the involved breast. For lesions in the inner or central quadrant, the internal mammary nodes were included within the tangential fields. A separate angled anterior field was employed to treat the supraclavicular and apical axillary nodes. A posterior field was added to supplement the dose to the midplane of the axilla. The involved breast received 4,600–5,000 rad, and the regional nodes received 4,400–5,000 rad delivered over a period of 4.5–5 weeks at the rate of 180–200 rad per fraction using 6-MeV photons. An additional boost of 1,500–2,000 rad was delivered to the area of the original primary tumor with interstitial iridium-192 or electrons.

Three chemotherapy regimens were employed. For CF, the regimen was cyclophosphamide at a dosage of 100 mg/m^2 orally on days 1–14 plus 5-fluorouracil at 600 mg/m^2 iv on days 1 and 8 of a 28-day cycle, administered for two cycles during concurrent radiotherapy. For CMF, the regimen was the same as that for CF plus methotrexate at 40 mg/m^2 iv on days 1 and 8 for six to 10 cycles after the completion of radiotherapy. For CMFP, the regimen was the same as that for CMF plus prednisone at 40 mg/m^2 orally on days 1–14 for six to 10 cycles after radiotherapy. The CF chemotherapy was initiated 3 weeks after axillary surgery at approximately the same time external beam irradiation was started.

Recently, Tormey et al. (*9*) reported the results of a randomized trial in metastatic breast cancer in which they observed a higher response rate and a significantly longer response duration and survival in patients who received CMFP, compared to those who received the standard CMF regimen. The CMFP patients received a higher percentage of the myelosuppressive agents than the CMF group. On the basis of this trial in advanced breast cancer, we modified our protocol and used CMFP as the adjuvant regimen in the majority of patients.

Initially, we administered 10 cycles of CMF after radiotherapy to complete 1 year of adjuvant chemotherapy. After the report by Tancini et al. (*10*) demonstrated no differences in either relapse-free survival or overall survival at 5 years for six versus 12 cycles of CMF after mastectomy, we reduced the duration of adjuvant chemotherapy administered after radiotherapy to six cycles.

During chemotherapy, we initially used a dose-reduction schedule based on the WBC count on days 1 and 8 of each treatment cycle. However, rather than administer a reduced dose of chemotherapy if leukopenia and/or thrombocytopenia were observed on day 1, we allowed a 1- to 2-week delay between cycles for recovery from myelosuppression, so that full doses of each agent could be administered. If myelosuppression was still observed after a delay ≤2 weeks, drug doses were reduced by 50% when the WBC count was 2,500–3,900/mm^3 and/or the platelet count was 75,000–99,000/mm^3.

However, given the known lympholytic effects of radiation with secondary lymphopenia, we changed these criteria for dose reduction in March 1984. Instead of reducing drug doses as a result of total WBC counts on days 1 and 8, we based drug doses on absolute granulocyte counts as follows. If granulocyte counts were ≥2,200/mm^3, 100% of the optimal doses of CMF were administered; if granulocyte counts were 1,800–2,199/mm^3 and platelet counts were ≥75,000/mm^3, 75% doses of CMF were given; and if granulocyte counts were 1,500–1,799/mm^3 and platelet counts were ≥75,000/mm^3, 50% doses were given. If granulocyte counts were <1,500 mm^3, CMF was not administered until the counts recovered. This dose-reduction schedule was utilized only if a 1- to 2-week delay failed to allow recovery of the granulocyte count to ≥2,200/mm^3. If nadir granulocyte counts were <500/mm^3 and/or nadir platelet counts were <50,000/mm^3, the doses of CMF in the next cycle were reduced by 25%.

Severe stomatitis and/or diarrhea led to a dose reduction of either methotrexate or 5-fluorouracil. In the presence of symptomatic cystitis secondary to cyclophosphamide, this drug was temporarily discontinued but was resumed with increased hydration as soon as this toxic effect had resolved. If hemorrhagic cystitis recurred, melphalan (4 mg/m^2 per day orally for 5 days) was substituted for cyclophosphamide in each cycle.

Dose calculation was performed using the method of Bonadonna and Valagussa (*7*). The amount of drug actually received was divided by the amount of drug planned for the two cycles of CF during radiotherapy and for the six cycles of CMF after radiotherapy. Mean values for both CF and CMF doses were calculated. Three dose levels were reported: level I, ≥85% of the optimal calculated doses; level II, 65%–84%; and level III, <65%.

The date of relapse was defined as the first date of suspicion of subsequently documented failure. Relapse was proven by biopsy whenever practical. Relapse-free survival and overall survival were defined as the time from axillary surgery to relapse or death, respectively. The Kaplan-Meier method was used to analyze relapse-free and overall survival.

RESULTS

The characteristics of the 96 patients receiving chemotherapy concurrently with definitive radiotherapy are summarized in table 1. The majority of the patients were premenopausal, with 1–3 positive axillary nodes. Only 15 patients had ≥4 positive nodes; 9 had negative nodes and negative estrogen receptors. Estrogen receptor status was not determined in 23 patients referred to our institution after the initial excisional biopsy was performed at another hospital.

The 96 patients began the CF portion of chemotherapy at a median of 24 days after axillary node dissection. The initiation of chemotherapy generally coincided with the start of external beam irradiation. The mean CF dose administered during concurrent radiotherapy was 95% (table 2). Eighty-three patients (86%) received level I doses of chemotherapy; 9, level II; and only 4, level III. The median time for drug delivery was 9 weeks. It was fre-

TABLE 1.—Patient characteristics[a]

Characteristic	No. of patients
Total	96
Menopausal status	
Premenopausal	63
Postmenopausal	33
Axillary node status	
Negative nodes	9
1–3 positive nodes	72
≥4 positive nodes	15
Primary tumor size	
T1	45
T2	51
Estrogen receptor status	
Positive	42
Negative	31
Unknown	23

[a] Mean time from axillary surgery to start of chemotherapy was 24 days (range, 11–64).

quently necessary to delay the second cycle of CF chemotherapy for 1–2 weeks to allow recovery of leukocyte or granulocyte counts, so that full doses of chemotherapy could be given during radiotherapy. Local skin reaction was mildly enhanced by concurrent radiotherapy and CF chemotherapy but did not delay the time of planned radiotherapy.

Eighty-three of the 96 patients have completed the planned six cycles of CMFP following definitive radiotherapy. Thirteen patients did not complete the six cycles of CMFP for the following reasons: 8 refused further treatment before completing the six cycles because of psychosocial reasons; melphalan was substituted for cyclophosphamide in 3 patients because of hemorrhagic cystitis; 1 patient developed symptomatic radiation pneumonitis and received only two cycles of CMF; and 1 died from an unrelated cause.

Table 2 summarizes the CMF drug doses given to the 83 evaluable patients who have completed six cycles of CMFP after radiotherapy. The mean CMF dose delivered over six cycles was 89% of the optimal calculated doses. Fifty-nine patients (71%) received level I doses, while only 6 received level III doses. A statistical comparison of the doses administered demonstrates that the doses of CMF given to the 56 patients receiving prednisone (mean CMF dose, 92%) were higher than those given to the 27 patients not receiving prednisone (mean CMF dose, 81%) ($P<.002$). The median time for delivery of the six cycles of CMF after radiotherapy was 26 weeks.

We analyzed separately the 8 patients who did not complete all six cycles of CMFP for psychosocial reasons (i.e., patient refusal to continue therapy). If the mean CMF doses for these 8 patients are analyzed on the basis of the actual number of cycles received, they received 91% of calculated doses. If the mean CMF doses are analyzed on the basis of the six scheduled cycles, 52% of the calculated doses were administered.

Cosmesis was evaluated in the 83 patients who completed all planned chemotherapy after definitive radiotherapy. At a median follow-up of 36 months after radiotherapy, cosmetic outcome was judged by physician evaluation to be excellent in 43% of the patients (no difference between the treated and untreated breasts) and good in 41% (minimal difference). In only 8 patients were the cosmetic results judged to be fair to poor, primarily due to increased fibrosis and reduction of breast size. Details of the cosmetic analysis and complications of radiotherapy have been previously described by Danoff et al. (*11*).

With a median follow-up of 36 months (range, 6–101) for all 96 patients, 16 relapses have been observed. The 4-year relapse-free survival for the whole group is 77%. None of the 9 patients with negative nodes has relapsed. Of the patients with 1–3 positive nodes, 11 have relapsed. The 4-year relapse-free survival for the group with 1–3 positive nodes is 78% (fig. 1). One of these patients had recurrence only in the breast and axilla, then underwent a mastectomy and remains disease free. This patient received two cycles of CF but only two cycles of CMF chemotherapy, then developed symptomatic radiation pneumonitis, which precluded any further chemotherapy. The 4-year relapse-free survival for the patients with ≥4 positive nodes is 58% (fig. 1); 5 patients have relapsed. One of these patients had recurrence in the axilla alone and is disease free after a full axillary dissection. The other 4 patients relapsed in distant sites. Thus, only 14 of 96 patients have had distant relapse to date; however, the median follow-up remains short.

Five of the 96 patients have died of breast cancer. The 4-year actuarial overall survival for all 96 patients is 93%. Figure 2 illustrates overall survival by nodal status. None of the node-negative, estrogen receptor-negative patients have died. Two of 72 patients with 1–3 positive nodes have died (4-yr overall survival, 97%). Three of 15 patients with ≥4 positive nodes have died (4-yr overall survival, 74%).

DISCUSSION

At the recent NIH Consensus Development Conference on Adjuvant Therapy for Breast Cancer, the panel concluded that when primary breast cancer is managed by conservative surgery and irradiation, full doses of chemotherapy should be administered (*6*). The panel's conclusions also noted that dose modifications to circumvent moderate and manageable toxicity may reduce the effectiveness of adjuvant therapy and should not be made. This conclusion was based on recent retrospective analyses that suggested improved results with the delivery of full-dose adjuvant chemotherapy in mastectomy patients with positive lymph nodes (*7,8*).

TABLE 2.—Distribution of patients according to dose level

	1st 2 cycles CF	Next 6 cycles CMFP
No. of patients	96	83
Mean % of optimal dose	95	89
Dose level		
I (≥85%)	83	59
II (65%–84%)	9	18
III (<65%)	4	6
No. of wk		
Median	9	26
Range	8–20	19–33

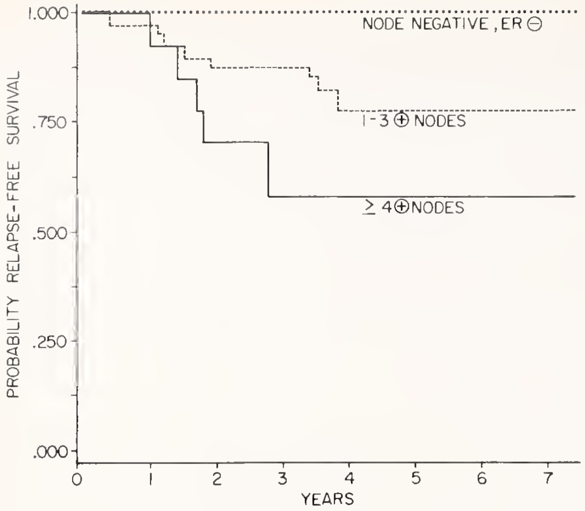


FIGURE 1.—Relapse-free survival by nodal status.

We have been able to administer 95% of the calculated doses of cyclophosphamide and 5-fluorouracil during concurrent definitive irradiation. Our decision to omit methotrexate during concurrent radiotherapy was based on observations in stage II and III patients, in whom the local skin reaction appeared to be exacerbated with concurrent CMF and radiotherapy. Thus, we recognize that the term full dose applies only to concurrent administration of cyclophosphamide and 5-fluorouracil plus radiotherapy, which was the original design of this protocol. Eighty-nine percent of the predicted CMF doses were delivered in the six cycles of CMFP chemotherapy following the completion of radiotherapy, without significant prolongation of the overall treatment plan. We have previously reported our results comparing patients treated with concurrent radiotherapy and CF chemotherapy followed by 6 months of CMF to a nonrandomized control cohort of matched patients who received CMF after mastectomy alone (*12*). In that study, there were no significant differences between patients receiving CMF after mastectomy or following definitive irradiation in the mean CMF doses delivered, the distribution of patients receiving level I doses, or the time of drug delivery. The present results expand our observations, and we conclude that full doses of adjuvant chemotherapy can be safely administered on schedule to patients receiving primary radiotherapy. Lippman et al. (*13*), using a relatively low-dose doxorubicin-cyclophosphamide regimen, also observed that there were no significant differences in the amount of chemotherapy administered to patients receiving irradiation, compared to a randomized control group of patients who had mastectomy.

The ability to deliver full doses of chemotherapy in our patients undergoing conservative surgery and radiation therapy may be related in part to the radiation technique employed. The complete avoidance of a direct or angled photon beam to treat the internal mammary nodes in the present series eliminates any dose to the bone marrow or the thoracic spine. It has been postulated by others (*14,15*) that the dose of radiation delivered to the thoracic spine with the direct or angled photon beam field may be responsible for the observed increase in hematologic toxicity.

Given the known lympholytic effects of radiation with secondary lymphopenia, we have also altered our dose-modification criteria for chemotherapy. Instead of changing drug doses based on total WBC count, we base doses on the absolute granulocyte count. This new dose-modification schedule is utilized only if a 1- to 2-week delay fails to allow granulocyte recovery to $\geq$2,200/mm^3. In addition, nadir counts are obtained and drug doses are reduced only if nadir granulocyte counts are $<$500/mm^3 and/or nadir platelet counts are $<$50,000/mm^3. Our observations are in accordance with those of Lippman et al. (*13*).

Patients initially received prednisone in our study as part of the CMFP regimen. They received higher doses of CMF, primarily due to the artificially elevated WBC counts on day 8 secondary to prednisone. This allowed the full administration of drug doses based on our original dose-modification criteria. However, we have recently deleted prednisone from our adjuvant regimen after a randomized Eastern Cooperative Oncology Group adjuvant trial demonstrated that the addition of prednisone to CMF produced no significant benefit but did increase toxicity (*16*).

It is not certain that substantially more aggressive chemotherapy regimens or doses will be associated with improved long-term relapse-free and overall survival, but this benefit is clearly a possibility (*7,8*). The 4-year relapse-free survival of 78% and the overall survival of 97% for patients with 1–3 positive nodes in our series are comparable to results reported by other investigators (*2–4,13,16*). Although median follow-up remains short, concurrent CF chemotherapy followed by six cycles of CMF provides at least equivalent disease control and survival to postmastectomy series in this subset. However, 4 of 15 of our patients with $\geq$4 positive nodes relapsed in distant sites, indicating that more intensive and innovative sequencing of chemotherapy and primary radiotherapy are indicated for this unfavorable subset. We are currently exploring the use of initial intensive chemotherapy with cyclophosphamide, doxorubicin, and 5-fluorouracil using dose escalation to produce nadir granulocyte counts of 500/mm^3 prior to the initiation of definitive radiotherapy for patients with $\geq$4 positive nodes.

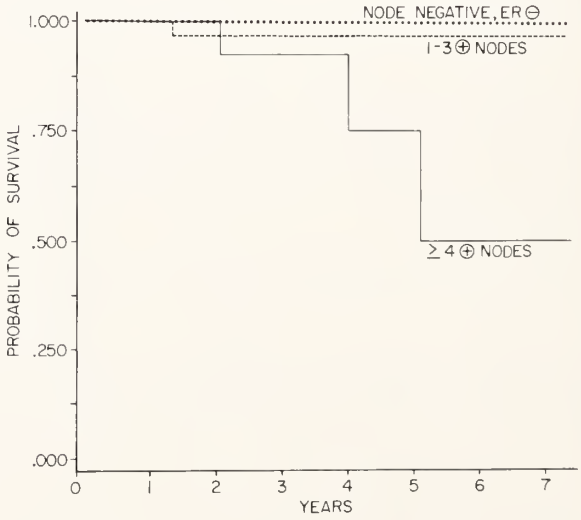


FIGURE 2.—Overall survival by nodal status.

REFERENCES

(*1*) Danoff B, Haller D, Glick J, et al: Conservative surgery and irradiation in the treatment of early breast cancer: A review. Ann Intern Med 102:634–642, 1985.

(*2*) Fisher B, Bauer M, Margolese R, et al: Five-year results of a randomized clinical trial comparing total mastectomy and segmental mastectomy with or without radiation in the treatment of breast cancer. N Engl J Med 312:665–673, 1985.

(*3*) Fisher B, Redmond C, Fisher ER, et al: Systemic adjuvant therapy in treatment of primary operable breast cancer: National Surgical Adjuvant Breast and Bowel Project experience. NCI Monogr 1:35–44, 1986.

(*4*) Bonadonna G, Valagussa P, Tancini G, et al: Current status of Milan adjuvant chemotherapy trials for node-positive and node-negative breast cancer. NCI Monogr 1:45–49, 1986.

(*5*) Lippman ME, Chabner BA: Editorial overview. NCI Monogr 1:5–10, 1986.

(*6*) National Institutes of Health Consensus Development Panel on Adjuvant Chemotherapy and Endocrine Therapy for Breast Cancer: Introduction and conclusions. NCI Monogr 1:1–4, 1986.

(*7*) Bonadonna G, Valagussa P: Dose-response effect of adjuvant chemotherapy in breast cancer. N Engl J Med 304:10–15, 1981.

(*8*) Hryniuk WM, Levine MN, Levin L: Analysis of dose intensity for chemotherapy in early (stage II) and advanced breast caneer. NCI Monogr 1:87–94, 1986.

(*9*) Tormey D, Gelman R, Band PR, et al: Comparison of induction chemotherapies for metastatic breast cancer: An Eastern Cooperative Oncology Group trial. Cancer 50:1235–1244, 1982.

(*10*) Tancini G, Bonadonna G, Valagussa P, et al: Adjuvant CMF in breast cancer: Comparative 5-year results of 12 versus 6 cycles. J Clin Oncol 1:2–10, 1983.

(*11*) Danoff B, Goodman R, Glick J, et al: The effect of adjuvant chemotherapy on cosmesis and complications in patients treated by definitive irradiation. Int J Radiat Oncol Biol Phys 9:1625–1630, 1983.

(*12*) Glick J, Danoff B, Haller D, et al: Integration of adjuvant chemotherapy with definitive radiotherapy for primary breast cancer. *In* Adjuvant Therapy of Cancer (Jones SE, Salmon SE, eds), vol IV. New York: Grune & Stratton, 1984, pp 291–300.

(*13*) Lippman ME, Edwards BK, Findlay P, et al: Influence of definitive radiation therapy for primary breast cancer on ability to deliver adjuvant chemotherapy. NCI Monogr 1:99–104, 1986.

(*14*) Abrams RA, Lichter A, Johnston-Early M, et al: Enhanced suppression of peripheral blood CFU_c and WBC and platelet counts by regional radiotherapy. Proc Am Assoc Cancer Res ASCO 20:144, 1979.

(*15*) Levine JF, Coleman CN, Cot RS, et al: The effect of postoperative and primary radiation therapy in delivered dose of adjuvant cyclophosphamide, methotrexate and 5-fluorouracil (CMF) chemotherapy in breast cancer. Cancer 53:237–241, 1984.

(*16*) Tormey DC, Gray R, Taylor SG, et al: Postoperative chemotherapy and chemohormonal therapy in women with node-positive breast cancer. NCI Monogr 1:75–80, 1986.

Comparison of Total Nodal Irradiation Versus Combined Sequence of Mantle Irradiation With Mechlorethamine, Vincristine, Procarbazine, and Prednisone in Clinical Stages I and II Hodgkin's Disease: Experience of the European Organization for Research and Treatment of Cancer[1]

P. Carde,[1,*] M. Hayat,[1] J. M. Cosset,[1] R. Somers,[1] J. M. V. Burgers,[1] W. Sizoo,[1] J. H. Meerwaldt,[1] A. Hagenbeek,[1] M. Monconduit,[1] E. van der Schueren,[1] J. Thomas,[1] A. Tanguy,[1] E. M. Noordijk,[1] J. Lustman-Marechal,[1] M. Schneider,[1] A. Cattan,[1] J. Michel,[1] B. De Pauw,[1] L. Verdonck,[1] M. Henry-Amar,[2] and M. Tubiana[1,3]

For the Lymphoma Cooperative Group of the European Organization for Research and Treatment of Cancer

ABSTRACT—**The H5 study of supradiaphragmatic Hodgkin's disease in clinical stages I–II consisted of two controlled trials adapted to patients considered to have either favorable or unfavorable characteristics, based on prognostic factors identified in two former studies by the European Organization for Research and Treatment of Cancer. Of 494 patients, 257 who were classified as having unfavorable prognosis qualified for the more intensive treatment and consequently were spared a staging laparotomy. They were randomized either to total nodal irradiation (TNI) (132 patients) or to treatment with mechlorethamine, vincristine, procarbazine, and prednisone (MOPP) alternated with mantle irradiation (MOPP×3-mantle irradiation-MOPP×3; 3M) (125 patients). In complete responders (96%), the 6-year relapse-free survival was 77% in the TNI arm and 91% in the 3M arm ($P=.02$). Relapses in the initially involved and irradiated mantle area were less frequent in patients started on MOPP. The 6-year actuarial total survival (TS) (TNI, 82%, and 3M, 89%; $P=.05$) appeared to favor the 3M arm, but this difference disappeared when patients dying from causes unrelated to cancer were excluded from analysis. In men≤40 years old, there was no difference in relapse-free survival, freedom from disease progression, or TS between the groups receiving TNI and 3M. Thus, TNI is a short and appealing treatment, especially because it preserves fertility. The same observation was true in women≤40 years old. In addition, even irradiation less than TNI, which is meant to spare the ovaries, provided a TS similar to that for 3M.—NCI Monogr 6:303–310, 1988.**

Although satisfactory results for therapy of CS I–II HD are achieved with various RT and/or CT programs (*1*), the optimal therapy and its toxic effects still need to be fully defined (*2*). Initial clinical presentation clearly influences outcome, as indicated by prognostic studies (*3,4*). From the outset, the EORTC Lymphoma Cooperative Group investigated how prognostic factors could be used to modulate the therapeutic strategy. This first became possible in 1977 after the H1 study (288 patients) and the H2 study (300 patients) were completed (*5–8*). In the H1 trial (1964), patient characteristics had been prospectively registered to determine which subgroups would best benefit from adjuvant CT. In the H2 trial (1972), a first attempt to modulate the treatment took into account the histological subtype (*8*). The preliminary analyses of the H2 trial demonstrated two important facts. 1) Prognostic factors were closely related to the type of therapy and could not be regarded as isolated (*1,4,5*). 2) The Sx was not beneficial in patients with poor prognostic factors but was highly informative in

---

ABBREVIATIONS: CS = clinical stage; HD = Hodgkin's disease; RT = radiotherapy; CT = chemotherapy; EORTC = European Organization for Research and Treatment of Cancer; Sx = staging laparotomy; TNI = total nodal irradiation; MOPP = mechlorethamine, vincristine, procarbazine, and prednisone; 3M = MOPP ×3-mantle irradiation-MOPP×3; MI = mantle irradiation; STNI = irradiation less than TNI; CR = complete remission; TRD = treatment-related death; FFP = freedom from disease progression; RFS = relapse-free survival (CR only); TS = total survival.

---

[1] This study was conducted by the European Organization for Research and Treatment of Cancer. The following members participated in the study: Institut Gustave-Roussy, Villejuif, France (*J. L. Amiel, P. Carde, J. M. Cosset, J. P. Droz, M. Hayat, M. Henry-Amar, G. Tchernia*, and *M. Tubiana*); Antoni van Leeuwenhoekhuis, Amsterdam, The Netherlands (*J. M. V. Burgers* and *R. Somers*); Rotterdamsch Radiotherapeutisch Instituut, Rotterdam, The Netherlands (*B. van der Werf-Messing, R. Qasim, W. Sizoo, J. H. Meerwaldt*, and *A. Hagenbeek*); Centre Henri Becquerel, Rouen, France (*M. Monconduit* and *H. Piguet*); Academisch Ziekenhuis Sint Rafael, Leuven, Belgium (*A. Drochmans, J. Thomas*, and *E. van der Schueren*); Centre François Baclesse, Caen, France (*J. S. Abbatucci* and *A. Tanguy*); University Hospital, Leiden, The Netherlands (*E. M. Noordijk*); Institut Jules Bordet, Brussels, Belgium (*J. Lustman-Marechal*); Centre Antoine Lacassagne, Nice, France (*M. Schneider*); Institut Jean Godinot, Reims, France (*A. Cattan*); Centre Hospitalier de Tivoli, la Louvière, Belgium (*J. L. Goffin* and *J. Michel*); St. Radboud University Hospital, Nijmegen, The Netherlands (*B. De Pauw* and *C. Haanen*); Academisch Ziekenhuis, Utrecht, The Netherlands (*J. G. Nyessen* and *L. Verdonck*); and Ziekenhuis Leyenburg's Gravenhage, La Haye, The Netherlands (*H. Kerkhofs*).

[2] Recipient of a grant from the European Organization for Research and Treatment of Cancer and grant CNAMTS-ARP No. 78-85 INSERM.

[3] We thank the data manager, Noëlle Dupouy.

[*] *Reprint requests:* Patrice Carde, M.D., Institut Gustave-Roussy, 39, rue Camille Desmoulins, 94805 Villejuif Cedex, France.

patients with favorable prognostic factors. The H5 program was the first in which surgical staging and therapy were tailored to patient characteristics leading to different strategies for patients with favorable or unfavorable prognosis. The strategy designed for treatment of the patients with favorable prognosis at the lowest predicted toxicity is not in the scope of this article. We present the results of the strategy for patients with unfavorable prognosis, which called for the more intensive treatment, in an attempt to provide the highest cure rate.

PATIENTS AND METHODS

Between January 1, 1977, and July 31, 1982, 494 previously untreated adult patients with CS I–II supradiaphragmatic HD were entered in the EORTC H5 protocol. On July 1, 1986, the mean follow-up was >5 years (64±23 mo). Characteristics of the patients with unfavorable prognosis are listed in table 1. This group was defined by the presence of at least one of four pejorative prognostic factors, which had been identified during two previous EORTC trials. These factors were age ≥40 years; erythrocyte sedimentation rate ≥70 mm/hour; histological subtype classified as "mixed cellularity" or "lymphocyte depletion"; and CS II disease without mediastinal involvement. The 257 patients with unfavorable prognosis at onset of disease were randomized to either TNI (132 patients) or 3M therapy (125 patients). The TNI consisted of MI at a dose of 40 Gy in 4 weeks. Boost doses to residual disease were optional up to 10 Gy. After a rest period, para-aortic, iliac, and inguinal areas plus spleen and splenic hilus were irradiated at the same doses as MI. In women ≤40 years old, the iliac and inguinal irradiation was optional and STNI was given unless oophoropexy had been performed or the patient was unconcerned about fertility. The 3M therapy consisted of three cycles of MOPP (*9*) before and after MI. At least 2 weeks were allowed for rest before and after MI. Radiation dose to the mantle was limited to 35 Gy. Pathological slides and lymphangiograms were reviewed by appointed review committees (table 1). Data were coded and prospectively registered at the statistics department of the Institut Gustave-Roussy. All patients were considered evaluable. Survival curves were plotted and tested using the log-rank method. Early relapses (within 3 mo of initial treatment) and disease progression during initial treatment resulted in similar outcome; patients with either disease

TABLE 1.—Characteristics of patients with unfavorable prognosis

Characteristic	Patients ($n=257$) No.	%
CS		
IA	76	30
IB	20	8
IIA	119	46
IIB	42	16
Age (yr)		
Mean ± SE: 37 ± 14		
<40	153	60
40–49	54	21
≥50	50	19
B symptoms	62	24
Sedimentation rate ≥70 mm/hr	63	25
Histology[a]		
Lymphocyte predominance	9	4
Nodular sclerosis	122	47
Mixed cellularity	123	47
Lymphocyte depletion	2	1
Not classified	1	1
Mediastinal involvement	102	40
Sex		
Male	180	70
Female	77	30

[a] Review committee.

TABLE 2.—Six-year results: patients with unfavorable prognosis

Results	% (No. of patients) Total ($n=257$)	TNI ($n=132$)	3M ($n=125$)	P value[a]
CR	96 (247)	95 (125)	98 (122)	NS
RFS ± SD	84 ± 3	77 ± 4	91 ± 3	.02
FFP ± SD	81 ± 3	73 ± 4	89 ± 3	.01
Survival ± SD	85 ± 3	82 ± 4	89 ± 3	.05
Tumor mortality ± SD	91 ± 2	90 ± 2[b] 89 ± 5[c]	92 ± 3	NS
TRD	2 (6)	3 (4)	2 (2)	NS
TRD with salvage therapy	0 (1)	1 (1)	0 (0)	NS
3-yr survival after relapse ± SD	63 ± 7[d]	66 ± 9	55 ± 14	NS

[a] NS = not significant.

[b] TNI + STNI (132 patients).

[c] TNI complete (62 patients).

[d] TNI + STNI in 33 patients; 3M in 17 patients.

TABLE 3.—Six-year results: sites of disease progression or relapse in patients with unfavorable prognosis[a]

Site of disease progression or relapse	Radiation field	All patients ($n=257$)	TNI ($n=132$)	3M ($n=125$)
Nodal, in-field	Mantle	1/9	1/7	0/2
	Inverted Y	0/2	0/2	—
Nodal, marginal	Mantle	1/2	1/2	0/0
	Inverted Y	0/0	0/0	—
Nodal, out-of-field[b]	Spleen	0/0	0/0	0/0
	Para-aortic	0/10	0/2	0/8
	Inguino-iliac	1/5	1/3	0/2
	Other	0/1	0/1	0/0
Visceral		7/11	4/9	3/2
Total		10/40	7/26	3/14
		4%/16%	5%/20%	2%/11%
Median interval from randomization to failure		20 mo	17 mo	32 mo
No. of relapses at >36 mo		13	6	7

[a] Unless otherwise specified, values = No. of patients with disease progression/No. with relapse.

[b] Splenic + para-aortic involvement in 1 case. Mantle in-field failure: TNI vs. 3M, $P=.07$. Mantle in-field + marginal failure: TNI vs. 3M, $P=.02$.

TABLE 4.—Six-year results: men ≤40 years old with unfavorable prognosis

	% (No. of patients)			
Results	Total ($n=104$)	TNI ($n=52$)	3M ($n=52$)	P value
CR	96 (101)	94 (50)	98 (51)	NS
RFS ± SD	84 ± 4	81 ± 6	87 ± 5	NS
FFP ± SD	80 ± 4	76 ± 6	85 ± 5	NS
Survival ± SD	90 ± 3	89 ± 5	91 ± 4	NS
Tumor mortality ± SD	92 ± 3	92 ± 4[a] 92 ± 5[b]	93 ± 4	

[a] TNI + STNI.
[b] TNI complete (37 patients).

TABLE 5.—Six-year results: women ≤40 years old with unfavorable prognosis

	% (No. of patients)			
Results	Total ($n=52$)	TNI[a] ($n=31$)	3M ($n=21$)	P value
CR	94 (49)	94 (29)	95 (20)	NS
RFS ± SD	89 ± 5	82 ± 7	100	0.05
FFP ± SD	84 ± 5	76 ± 8	95 ± 5	0.05
Survival ± SD	90 ± 4	90 ± 6	90 ± 7	NS
Tumor mortality ± SD	92 ± 4	93 ± 5	90 ± 7	NS

[a] All patients except 3 women received STNI.

status were classified as having "no CR achievement." The patients with TRD were included as having treatment failure (either no CR achieved or relapse) and as deaths from HD. Patients who died from unrelated disease or second cancer without clinical or autopsy evidence of HD were censored in the data relating to FFP, RFS, and tumor mortality at the time of death but were included in the TS curves. The curve of FFP events includes failure to achieve CR. The actuarial curves start from the date of randomization.

RESULTS

Complete remission.—Of 257 patients, 247 (96%) achieved clinical CR (table 2). Four of the 10 patients who did not attain CR had disseminated disease and died (2 in each arm).

Relapse-free survival in complete responders.—The overall RFS at 6 years was 84% (table 2). The RFS was significantly higher in the 3M group than in the TNI group (91% vs. 77%, respectively; $P = .02$). The relapse patterns differed according to the therapy given (table 3). After 3M, 10 of 14 relapses occurred in the nonirradiated infradiaphragmatic nodal areas: 8 in para-aortic nodes and 2 in inguino-iliac nodes. Isolated splenic relapse was not observed. After TNI, 9 patients had supradiaphragmatic failure (3.5% of patients; 20% of failures) in or marginal to the radiation field versus 2 patients in the 3M arm. In the TNI arm, there were 9 visceral relapses versus only 2 in the 3M arm. Differences between TNI and 3M were smaller or absent in patients ≤40 years old, especially in men (tables 4 and 5).

Freedom from disease progression.—The 6-year FFP was higher in the 3M arm ($P = .01$) (table 2 and fig. 1), in keeping with the higher CR and RFS rates. Salvage therapy achieved a long-lasting CR in 38 of 50 patients with relapse (76%), in more patients in the TNI group (82%) than in the 3M group (65%).

Treatment-related deaths and second malignancies.—Overall, 3% of the patients (7 of 257) died from TRD (table 6). In the 3M arm, TRD was due to opportunistic infections. In the TNI arm, 3 deaths were related to radiation mediastinitis and 1 or 2 to radiation enteritis. Deaths from second malignancy occurred in 8 patients (table 7). Among patients without relapse, acute nonlymphoblastic leukemia developed in 1 patient in each group and non-Hodgkin's lymphoma developed in 1 patient in the TNI group.

Survival.—The 6-year TS was 85%. It was significantly higher ($P = .05$) in the 3M arm (89%) compared to the TNI arm (82%) (table 2 and fig. 2). However, survival was iden-

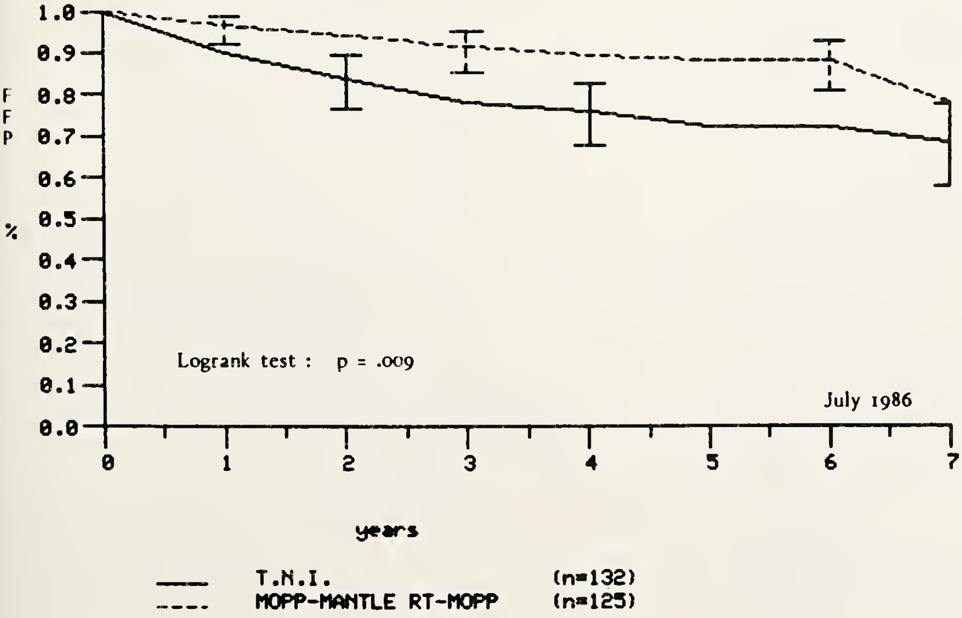


FIGURE 1.—Comparison of FFP after TNI or 3M therapy in 257 patients with CS I–II HD with unfavorable prognosis.

TABLE 6.—Six-year results: TRD in patients with unfavorable prognosis[a]

Patient No.	Treatment	Interval (mo)	Initial age (yr)	Cause	Status at death
1	TNI	48	65	Pulmonary fibrosis, heart failure	CR
2	TNI	22	16	Mediastinitis, pericarditis, pleural effusion	Relapse
3	TNI	14	25	Pulmonary embolism (mediastinitis, radiation pleurisy	CR
4	TNI	67	61	Septic shock after surgery for radiation enteritis	CR (autopsy)
5	3M	2	37	Pulmonary infection during MOPP hypoplasia (radiation pneumonitis, very bulky mediastinum)	CR
6	3M	14	68	Herpes zoster, opportunistic infection	CR
7	TNI[b]	22	31	MOPP; intestinal necrosis complicating surgery for appendicitis	Relapse

[a] Does not include deaths from 2nd malignancies.

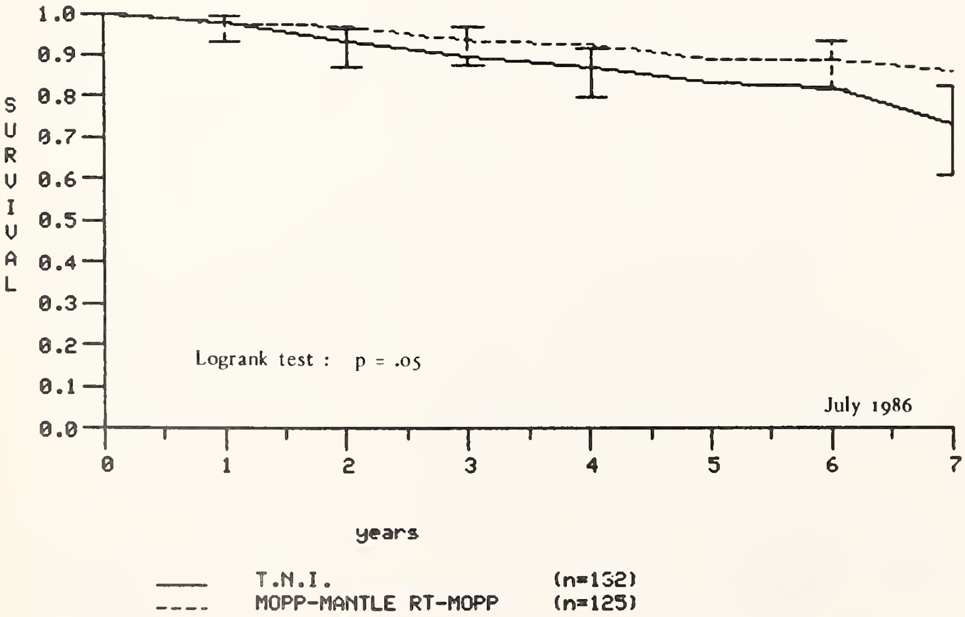
[b] TRD associated with salvage therapy.

TABLE 7.—Six-year results: distribution of 37 deaths in patients with unfavorable prognosis

Cause	No. of patients TNI	 3M
Hodgkin's disease, progression	2	2
Hodgkin's disease, relapse	4	4
TRD	4	2
TRD with salvage treatment	1	0
Second malignancy	6	2
Unrelated to cancer	8	2

tical in patients ≤40 years old (tables 4 and 5), even in women who were treated only with STNI to spare the ovaries (table 8). Overall, 19 patients (7%) died either of HD or TRD. Survival after relapse was not significantly higher in patients initially treated with TNI. Of the 10 patients (4%) who died for reasons unrelated to HD or to treatment, 8 were in the TNI group (table 7). When deaths unrelated to cancer and those from second cancer were censored, the tumor mortality (tables 2, 4, 5, and 8 and fig. 3) was similar in all subgroups of the TNI and 3M arms.

Treatment duration, tolerance, and complications.—Median duration of treatment was significantly shorter in the TNI arm (107 vs. 210 days). In 48% of the cases, RT to an inverted-Y field was not given or completed. This occurred mainly in women who wished to preserve their fertility (28%), but it also occurred due to protocol violation (20%). Tables 2, 4, and 5 and figure 3 show tumor mortality for TNI and STNI. An excess of inguino-iliac failures was demonstrated in subgroups of patients who did not receive complete TNI (TNI complete, 0%; all STNI, 3%; STNI in women ≤40 yr old, 6%). There was a trend for



Logrank test : p = .05

July 1986

FIGURE 2.—Comparison of survival after TNI or 3M therapy in 257 patients with CS I–II HD with unfavorable prognosis.

Table 8.—Six-year results according to treatment by stage/B symptoms in patients with unfavorable prognosis

CS	No. of patients			% CR			% RFS ± SD			% FFP ± SD			% TS ± SD			% Tumor mortality ± SD			
	All	TNI + STNI	3M	All	TNI + STNI	3M	All	TNI + STNI	3M	All	TNI + STNI	3M	All	TNI + STNI	3M	All	TNI + STNI	TNI[a]	3M
IA	76	37	39	99	100	97	81±6	68±9	92±7	79±6	68±9	90±8	84±5	74±8	95±4	91±4	86±6	76±13	97±3
IB	20	9	11	95	89	100	85±7	73±16	100	85±8	65±17	100	86±10	67±21	100	100	100	100	100
IIA	119	62	57	97	95	98	86±4	85±5	87±5	83±4	81±5	85±5	86±3	87±5	85±5	91±3	95±3	97±3	87±5
IIB	42	24	18	90	88	94	75±7	75±10	75±11	68±7	65±10	71±11	81±6	82±8	80±10	87±6	87±7	92±8	86±10

[a] TNI complete (IA, 18 patients; IB, 3; IIA, 33; IIB, 12).

decreased RFS and FFP in patients who were not given complete TNI. This was not the case, however, in patients ≤40 years old (tables 4 and 5). Because of hematologic intolerance, there was a prolonged interval between the completion of MI and the end of the last three cycles of MOPP. Treatment was stopped due to intolerance in 13 patients receiving 3M. The MOPP drug doses were believed to be of significant importance (*10*) and were prospectively registered. Mean doses of mechlorethamine, vincristine, and procarbazine were decreased progressively during the first and second series of MOPP: 87% of the theoretical dose at cycle 1, 78% at cycle 2, 72% at cycle 3, 74% at cycle 4, 61% at cycle 5, and 64% at cycle 6. Overall, 72% ± 18% of the theoretical dose was given for mechlorethamine; 64% ± 20% for vincristine; and 68% ± 22% for procarbazine.

For comparative purposes, results are given according to the Ann Arbor staging system in each treatment group (table 8). The pejorative characteristics of the H5 population with unfavorable prognosis must be considered.

DISCUSSION

Staging Laparotomy and Prognostic Factors

In the H5 program, workup and therapy were tailored to pretreatment characteristics. On the basis of the prognostic factors formerly identified, patients were entered in two controlled trials. The 257 patients with unfavorable characteristics and high likelihood of infradiaphragmatic involvement [>35% (*11*)] could be spared the inconvenience (*12*) of Sx because they were all to receive intensive treatment. This decision is in accordance with the Ann Arbor Committee recommendation that Sx should be performed only if management relies on its results (*13*). Because of a similar strategy using prognostic factors, there has been a change in policy regarding Sx at Stanford University, CA (*14*). Even when patients with poor prognosis have had laparotomy that showed no infradiaphragmatic involvement, the relapse rate (>50%) has been unacceptable (*8,11,15,16*) when irradiation was limited to the mantle, para-aortic, and splenic fields (STNI). Therefore, these patients were offered a more intensive treatment (H5); the randomization between TNI and combination therapy (3M) reflects the uncertainty about which modality was best. With the use of the H5 criteria, significant progress has been made in this group with unfavorable prognosis, in both RFS and survival; this is shown by comparison with the H1 and H2 results. Prognostic factors other than those used in the H5 trial have been reported as relevant (*14,17–19*). In our series, it was found that bulky mediastinal disease was correlated to other prognostic factors (*11,20*) and that it only influenced RFS in patients treated with RT alone. However, within the subgroup of patients with involved mediastinum, 10% had a mediastinum-thorax ratio >0.50. In our experience, the RFS and TS of those patients were poor regardless of the initial treatment (*11*), as noted by others (*21–24*). Nevertheless, prior to the initiation of the present study in 1977, there were few attempts to tailor individual therapy to prognostic factors (*25*); only systemic symptoms were used (*14,19*).

Therapeutic Results

Complete remission.—Failure to achieve CR was of concern both quantitatively (10 patients, 4%) and for survival; 6 of 10 patients with disease progression died, compared to

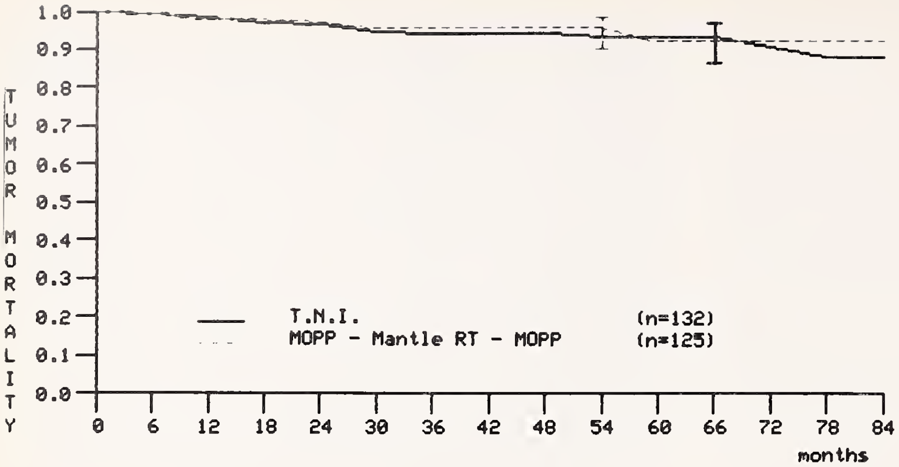


FIGURE 3.—Comparison of tumor mortality after TNI or 3M therapy in 257 patients with CS I–II HD with unfavorable prognosis.

only 8 patients overall who died after relapse due to HD. Initial CT did not decrease the number of patients with disseminating disease that led to death.

Relapse-free survival, freedom from disease progression, and pattern of relapse.—The RFS and the FFP were significantly higher in the combined modality arm (table 2 and fig. 1). The results were not unusually poor in the TNI arm (*13,14,18*), especially considering the group with poor prognosis that was selected in the H5 trial. In patients with CS IB and IIB disease, results in the group receiving RT plus CT appeared markedly better than in the RT arm in the EORTC study. Early administration of CT may be of importance in these patients with aggressive disease, as suggested in other studies (*23*). The modalities of relapse differed according to treatment (table 3). Incidence of nodal in-field or marginal relapse appeared to be lower in the 3M arm, suggesting some additive effect of the combined modality therapy (CT–RT–CT). This result confirms previous EORTC observations (*5,7*). Conversely, para-aortic and inguino-iliac relapses accounted for most of the failures in the 3M arm, suggesting that six cycles of MOPP are less effective than RT to an inverted-Y field for sterilization of nodal occult disease (table 3). Most of the relapses that occurred in patients with RT to an inverted-Y field plus the spleen were observed in the TNI arm in those who had incomplete irradiation. In the 3M arm, no isolated splenic failure was observed and the incidence of disseminated relapses was markedly lower than in the TNI arm (2 vs. 9 cases).

Survival.—In CS I–II HD, the observation of improved survival (fig. 2) from combining MOPP and RT, (at 6 yr, 89% for 3M vs. 82% for TNI; $P = .05$) should be interpreted with care. None of the other studies comparing RT and RT plus MOPP showed significance in terms of survival (*21–23,26,27*), except the Stanford studies, for CS IIIA disease (*14*). Absence of a significant difference in other studies, some of which were not randomized, may be related to enrollment of patients with a better prognosis: laparotomy-staged patients, with no selection for poor prognostic factors. This is reflected by a slightly higher survival in the RT-alone groups from these studies than in our TNI arm. These results prompted us to analyze the patient subsets that benefited most from the combined modality therapy. A survival benefit from CT plus RT was observed for CS IA and IB but not for CS IIA and IIB (table 8). In men and women ≤40 years old (tables 4 and 5), the FFP advantage of CT plus RT was not translated into survival, probably due to better efficacy and lower toxicity of salvage treatment in the RT arm. It should be emphasized that most patients who experienced an "unrelated death" due to intercurrent causes were enrolled in the TNI arm and were >40 years old. No difference in tumor mortality was observed between TNI or STNI and 3M in any subgroup (fig. 3). Older patients appeared to be at higher risk for death from HD, as previously reported (*28*). Therefore, further updating and continuous follow-up of this EORTC study will be mandatory to evaluate the role of CT in any subgroup (*29*). Compliance to treatment had a limited, although definite, influence on survival. In the TNI arm, there was a trend toward lower survival and lower tumor mortality (tables 2, 4, 5, and 8) in patients with incomplete TNI; there was a similar trend for RFS and FFP. In patients ≤40 years old, even the crude 6-year survival times in men and in women who received STNI to spare the ovaries were 89% and 88%, respectively, compared to 91% and 90%, respectively, with 3M. In the 3M arm, mean doses of mechlorethamine, vincristine, and procarbazine were prospectively recorded for 93 patients. In the 44 patients who could not receive >60% of the theoretical dose, the 6-year survival was 90% versus 98% in other patients. The impact of drug intake on survival is not mediated through CR achievement, as almost all patients achieved CR; dose-response effects in HD were first demonstrated in patients with stage IIIB or IV HD (*10*).

Treatment-related deaths, second malignancies, and deaths unrelated to cancer.—There were 7 deaths (3%) related to first treatment and TRD associated with salvage therapy (tables 6 and 7). In 4 of these patients, death was associated with mediastinal radiation damage. Radiation enteritis was also associated with TRD in 2 patients. Among second malignancies, acute nonlymphoblastic leukemia represents the major concern for physicians (*30–32*). Our follow-up of only 6.5 years does not allow any comments. Of 10 unrelated deaths (1 from suicide and 6 from unrelated diseases), 3 were sudden and unexplained (at ages 22, 31, and 61 yr) and 1 occurred after major coronary surgery. It is difficult to rule out myocardial radiation damage as a cause (*13,33*).

CONCLUSIONS

In this group with the poorest prognosis, which was divided as in the H5 trial, FFP and survival continuously improved in three consecutive EORTC trials. This result confirms the validity of the prognostic factors chosen for the H5 trial (*34*). These prognostic factors have been further refined (*4,15*). It also justifies the policy of dividing the patient population to intensify treatment for some of them. The combined CT–RT modality resulted in higher RFS and FFP than did RT alone, but there was no definite survival advantage. The absence of survival difference in patients ≤40 years old demonstrates that TNI (or STNI) and 3M both are valuable treatments. It allows consideration of fertility and second cancer risks as important issues for treatment decision in younger patients with unfavorable CS I–II HD.

REFERENCES

(*1*) KAPLAN HS: Hodgkin's Disease, 2nd ed. Cambridge, MA, London: Harvard Univ Press, 1980.

(*2*) LONGO DL, YOUNG RC, DEVITA VT JR: Chemotherapy for Hodgkin's disease: The remaining challenges. Cancer Treat Rep 66:925–936, 1982.

(*3*) TUBIANA M, ATTIE E, FLAMANT R, et al: Prognostic factors in 454 cases of Hodgkin's disease. Cancer Res 31: 1801–1810, 1971.

(*4*) TUBIANA M, HENRY-AMAR M, VAN DER WERF-MESSING B, et al: A multivariate analysis of prognostic factors in early stage Hodgkin's disease. Int J Radiat Oncol Biol Phys 11:23–30, 1985.

(*5*) TUBIANA M, VAN DER WERF-MESSING B, LAUGIER A, et al: Survival after recurrence: Prognostic factors and spread patterns in clinical stages I and II of Hodgkin's disease. Natl Cancer Inst Monogr 36:513–530, 1973.

(*6*) TUBIANA M, HENRY-AMAR M, HAYAT M, et al: Long-term results of the EORTC randomized study of irradiation and vinblastine in clinical stages I and II of Hodgkin's disease. Eur J Cancer 15:645–657, 1979.

(*7*) RADIOTHERAPY-CHEMOTHERAPY GROUP OF EORTC: A randomized study of irradiation and vinblastine in stages I and II of Hodgkin's disease. Preliminary results. Eur J Cancer 8:353–362, 1972.

(*8*) TUBIANA M, HAYAT M, HENRY-AMAR M, et al: Five year results of the EORTC randomized study of splenectomy and spleen irradiation in clinical stages I and II of Hodgkin's disease. Eur J Cancer 17:355–363, 1981.

(*9*) DEVITA V JR, SIMON R, HUBBARD S, et al: Curability of advanced Hodgkin's disease with chemotherapy. Long-term follow-up of MOPP treated patients at the National Cancer Institute. Ann Intern Med 92:587–595, 1980.

(*10*) CARDE P, MACKINTOSH FR, ROSENBERG SA, et al: A dose and time response analysis of the treatment of Hodgkin's disease with MOPP chemotherapy. J Clin Oncol 1:146–153, 1983.

(*11*) TUBIANA M, HENRY-AMAR M, HAYAT M, et al: The EORTC treatment of early stages of Hodgkin's disease: The role of radiotherapy. Int J Radiat Oncol Biol Phys 10:197–210, 1984.

(*12*) COLEMAN CN, MCDOUGALL IR, DAILEY MO, et al: Functional hyposplenia after splenic irradiation for Hodgkin's disease. Ann Intern Med 96:44–47, 1982.

(*13*) CORNBLEET MA, VITOLO U, ULTMANN JE, et al: Pathologic stages IA and IIA Hodgkin's disease: Results of treatment with radiotherapy alone (1968–1980). J Clin Oncol 3:758–768, 1985.

(*14*) ROSENBERG SA, KAPLAN HS: The evolution and summary results of the Stanford randomized clinical trials of the management of Hodgkin's disease: 1962–1984. Int J Radiat Oncol Biol Phys 11:5–32, 1985.

(*15*) TUBIANA M, HENRY-AMAR M, HAYAT M, et al: Prognostic significance of the number of involved areas in the early stages of Hodgkin's disease. Cancer 54:885–894, 1984.

(*16*) HAYBITTLE JL, EASTERLING MJ, BENNETT MH, et al: Review of British National Lymphoma Investigation of Hodgkin's disease and development of prognostic index. Lancet 1:967–972, 1985.

(*17*) SUTCLIFFE SB, GOSPODAROWICZ M, BUSH RS: Mediastinal involvement by Hodgkin's disease and the implications for management for those patients with local or loco-regional disease. Hematol Oncol 2:74–76, 1984.

(*18*) BERGSAGEL DE, ALISON RE, BEAN HA, et al: Results of treating Hodgkin's disease without a policy of laparotomy staging. Cancer Treat Rep 66:717–731, 1982.

(*19*) ROSENBERG SA: The current status of the Stanford randomised clinical trials of the management of Hodgkin's disease. *In* Malignant Lymphomas and Hodgkin's Disease: Experimental and Therapeutic Advances (Cavalli F, Bonadonna G, Rozencweig M, eds). Boston: Martinus Nijhoff, 1985, pp 281–292.

(*20*) COSSET JM, HENRY-AMAR M, CARDE P, et al: The prognostic significance of large mediastinal masses in the treatment of Hodgkin's disease. The experience of the Institut Gustave-Roussy. Hematol Oncol 2:33–43, 1984.

(*21*) NISSEN NI, NORDENTOFT AM: Radiotherapy versus combined modality treatment of stage I and II Hodgkin's disease. Cancer Treat Rep 66:799–803, 1982.

(*22*) ANDERSON H, DEAKIN DP, WAGSTAFF J, et al: A randomised study of adjuvant chemotherapy after mantle radiotherapy in supradiaphragmatic Hodgkin's disease PS IA-IIB: A report of the Manchester Lymphoma Group. Br J Cancer 49:695–702, 1984.

(*23*) LESLIE NT, MAUCH PM, HELLMAN S: Stage IA to IIB supradiaphragmatic Hodgkin's disease. Long term survival and relapse frequency. Cancer 55:2072–2078, 1985.

(*24*) VELENTJAS E, BARRET A, MCELWAIN TJ, et al: Mediastinal involvement in early stage Hodgkin's disease. Eur J Cancer 16:1065–1068, 1980.

(*25*) YOUNG RC, LONGO DL, GLATSTEIN E, et al: The current status of NCI trials in Hodgkin's disease. *In* Malignant Lymphomas and Hodgkin's Disease: Experimental and Therapeutic Advances (Cavalli F, Bonadonna G, Rozencweig M, eds). Boston: Martinus Nijhoff, 1985, pp 293–298.

(*26*) HAGEMEISTER FB, FULLER LM, VELASQUEZ WS, et al: Stage I and II Hodgkin's disease: Involved-field radiotherapy versus extended-field radiotherapy versus involved-field radiotherapy followed by six cycles of MOPP. Cancer Treat Rep 66:789–798, 1982.

(*27*) FREI E III, LUCE JK, GAMBLE JF, et al: Combination chemotherapy in advanced Hodgkin's disease. Induction and maintenance of remission. Ann Intern Med 79:376–387, 1973.

(*28*) AUSTIN-SEYMOUR MM, HOPPE RT, COX RS, et al: Hodgkin's disease in patients over sixty years old. Ann Intern Med 100:13–18, 1984.

(*29*) TUBIANA M, COSSET JM, CARDE P, et al: The contribution of clinical trials to the treatment of patients with early stages of Hodgkin's disease. Drugs Exp Clin Res 12: 105–112, 1986.

(*30*) HENRY-AMAR M: Second cancers after treatment in two successive cohorts of patients with early stages of Hodgkin's disease. *In* Malignant Lymphomas and Hodgkin's Disease: Experimental and Therapeutic Advances (Cavalli

F, Bonadonna G, Rozencweig M, eds). Boston: Martinus Nijhoff, 1985, pp 417–428.

(*31*) Carde P, Henry-Amar M, Tubiana M, et al: No increased incidence of second leukemias in patients treated with vinblastine alone or associated with procarbazine following radiotherapy in 2 successive EORTC controlled trials (1964–1976) in clinical stages I–II Hodgkin's disease. Proc ASCO 4:212, 1985.

(*32*) Coleman CN, Williams CJ, Flint A, et al: Hematologic neoplasia in patients treated for Hodgkin's disease. N Engl J Med 297:1249–1252, 1977.

(*33*) Annest LS, Anderson RP, Li W, et al: Coronary artery disease following mediastinal radiation therapy. J Thorac Cardiovasc Surg 85:257–263, 1982.

(*34*) Tubiana M, Henry-Amar M, Burgers MV, et al: Prognostic significance of ESR in clinical stages I and II Hodgkin's disease. J Clin Oncol 2:194–200, 1984.

Discussion Summary

Michael A. Friedman[1,*] and Harry Bartelink[2]

Combinations of radiation and chemotherapy are being investigated increasingly in both preclinical and clinical settings. These CM approaches have a two-fold purpose: to enhance local tumor destruction (increase local control rate) and to lower the total body burden of tumor (increase cure rate). The 11 papers in this session address various aspects of such attempts. The subsequent discussion period focused on three categorical questions.

First, is local control a meaningful clinical end point for CM studies, and in which tumor types has it been achieved? Dr. Glatstein suggested that, despite therapeutic improvements in diseases like head and neck and cervix cancers for most common adult cancers, any gain in local tumor clearance is negated by lack of control of systemic disease. Adequate regional antitumor effect occurs in the face of rapid demise due to metastatic disease. Perhaps, he postulated, the ability to clear local tumor has been overemphasized. Offering a contrasting position, an investigator stressed that CM advances have been made in both small and non-small cell lung cancer that he believes are meaningful. Careful pathologic confirmation of tumor necrosis within the treatment field provides important data. No general statement was framed for the multiple potential opportunities that were believed to exist for research in this area.

A second related issue is the determination of what the optimal design for a CM clinical trial should be. Several speakers described how chemotherapy can enhance normal as well as neoplastic tissue radiosensitivity. An ideal CM treatment would selectively kill tumor cells, and yet many CM approaches result in severe local toxicity. To test the therapeutic gain of such approaches, one will be confronted with a therapeutic dilemma. What should be the radiation dose in the CM and control arms? It might be that dose of radiation therapy alone that produces a comparable level of critical normal tissue injury, even if this means a higher total dose being delivered (than that in the chemotherapy plus radiation therapy arm).

Clinically determining if the dose–response curve has been modified by an approach is not a simple task for investigators. Unfortunately, the majority of clinical experiments are not designed to provide meaningful dose–response data.

Recognizing these methodologic difficulties, the crucial study end points for a CM trial seem to be: 1) local control rate: pathologic confirmation and sufficient follow-up (e.g., 2 or 5 yr) for confident assessment of the permanence of a regional effect; 2) systemic control rate: the duration of survival, disease-free time, and pattern of failure; and 3) local toxicity: qualitative and quantitative assessments of histologic and physiologic normal tissue impairment. For example, in patients with glioblastoma multiforme receiving radiation therapy plus misonidazole, a significant toxicity like brain necrosis may be obscured in the simple survival estimates which reflect the poor prognosis of this tumor. For pediatric patients with Ewing's sarcoma, a CM treatment may eradicate that tumor acutely but may induce secondary cancers which become apparent because the patients live sufficiently long.

Clearly, there is insufficient understanding of the cell biology impact of CM therapy. Does an increase of a few logs of cell killing initially affect overall tumor viability, with respect to micrometastases and the emergence of potential therapy resistance?

A third general area of discussion dealt with the selection of ideal drugs for CM trials. The general theme of insufficient or inefficient linkage of preclinical to clinical information was reiterated. Specific clinical areas of interest discussed included head and neck, primary brain, and gastrointestinal tumors. The complicated biologic heterogeneity of patients and tumors makes it difficult to generalize, even when our attention is limited to a single histologic entity.

Considerable discussion attended the use of mitomycin plus radiation therapy in head and neck cancer. It was proposed that bioreductive alkylators (like mitomycin) may be particularly useful adjuncts to radiation therapy, generally, and mitomycin for hypoxic cells, specifically. Such differential cytotoxicity may be more evident in vitro than in vivo, and the cellular pharmacology of this phenomenon is unknown.

On a related topic, there are a raft of questions concerning the best manner of using FUra with radiation therapy. Neither the proper dose and schedule of FUra nor radiation therapy have been defined, so that the optimal CM combination can only be surmised. Moreover, as was pointed out, there may be no advantageous intracellular concentration of FUra (or metabolites) in normal versus tumor tissue despite positive clinical results. The initial leads could be exploited in gastrointestinal cancer with FUra and radiation therapy, even with this limited degree of understanding.

In general, it was recognized that some advances in the understanding of CM treatments have been made. However, the need for further progress is considerable.

ABBREVIATIONS: CM = combined modality; FUra = 5-fluorouracil.

---

[1] Clinical Investigations Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[2] Department of Experimental Radiotherapy, The Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Amsterdam.

[*] *Reprint requests:* Michael A. Friedman, M.D., Clinical Investigations Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Landow Bldg., Rm. 4A04, National Institutes of Health, Bethesda, MD 20892.

Combined Modality Treatment—II

Keynote Address: Platinum-Radiation Interactions [1]

Evan B. Douple [2], *

ABSTRACT—**The important chemotherapeutic agent cisplatin is currently being combined with radiation therapy (RT) in clinical protocols intended to exploit the potential for this drug to potentiate radiation-induced tumor cell kill. This paper reviews the reports from preclinical studies leading to the design of combined modality protocols and describes the effects produced when platinum complexes are combined with RT. Two interactions that are receiving considerable attention since they might produce an improved therapeutic ratio are the radiosensitization of hypoxic cells and post-RT potentiation of cell kill. This latter effect might include the inhibition of recovery from RT-induced potentially lethal or sublethal damage. However, platinum-radiation interactions are complex and probably include several mechanisms that are unknown at this time. The potential for platinum complexes will be especially promising if results of ongoing phase III combined modality trials show them to be efficacious, since it is unlikely that current protocol designs are optimal. Furthermore, second-generation platinum analogs or other metal complexes designed as potentiators of RT may prove to be more interactive with RT.—NCI Monogr 6:315–319, 1988.**

EARLY OBSERVATIONS OF INTERACTIONS

As phase I clinical trials of cisplatin were beginning and before this parent Pt complex became established as an important chemotherapeutic agent with promising broad-spectrum activity in a variety of tumors, two reports suggested that the combination of cisplatin with ionizing radiation might result in interactions. In 1971, Zak and Drobnik (*1*) observed that post-RT lethality in mice was enhanced or reduced depending on the timing between the administration of cisplatin and RT. In 1974, Wodinsky et al. (*2*) reported that the combination of cisplatin chemotherapy and RT produced a synergistic therapeutic effect on P388 lymphocytic leukemia in mice. However, it was quantitative studies of the survival of bacterial spores by Richmond and Powers (*3*) in 1976 that demonstrated potentiation of RT-induced lethality that was especially enhanced under hypoxic conditions during RT, although some radiopotentiation of aerated spores was reported. These investigators showed that killing of bacterial spores by Pt plus RT involved complex processes including free radicals and free electron scavenging. Two years later, a small amount of radiosensitization of both hypoxic (*4*) and oxygenated (*5*) cultured mammalian cells by cisplatin was reported. These effects obtained with cisplatin were extended to include another Pt coordination complex (*6*), and the effects observed in cultured cells were produced in animal tumor cells irradiated in situ (*7*).

RESULTS OF IN VITRO STUDIES

During the past decade, cisplatin and other Pt complexes, when combined with RT, have been shown to produce cell kill greater than that expected from the additive effects of the two agents (*8–13*). Operationally, at least two types of potentiation are observed: potentiation before and after RT. The first type is a radiosensitization; i.e., enhanced cell killing produced when the Pt complexes are administered before RT. Results of studies in bacterial systems suggest that cisplatin operates in part through reactive free radicals, including the hydrated electron and the hydroxyl radical, in part through the interaction of RT-induced reactive Pt (I) intermediates, and in part through alteration of Pt (II)-DNA binding during RT (*14*). The results of these bacterial studies suggest that cisplatin appears to be more effective as a radiation potentiator in free solution than when it is bound to cellular components. Irradiated solutions of cisplatin (*14*) and certain other Pt complexes (*15*) are more toxic than unirradiated solutions, an effect hypothesized to be a result of free radical formation and subsequent reaction to Pt (I) intermediates.

In mammalian cells, it is difficult to compare the radiosensitization produced by cisplatin to that produced by misonidazole on an equimolar basis since cisplatin produces significant cytotoxicity at concentrations greater than about 10 μM. However, at these low concentrations, cisplatin does modify the terminal slope of the radiation survival curves under hypoxic conditions during RT. The hypoxic cell radiosensitization by a variety of metal complexes reported to date suggests that this phenomenon is probably not predicted by an electron affinity relationship such as has been correlated to the activities of the nitroimidazole class of hypoxic cell radiosensitizers.

In the second type of interaction, post-RT potentiation occurs when the drug is added after RT, at a time when free radical-mediated radiosensitization would not be expected to occur. This post-RT potentiation by cisplatin was first demonstrated in V79 cells (*16*) and later in rat hepatoma cells (*17*). Subsequently, similar post-RT potentiation was reported for other Pt complexes (*18*), including second-generation complexes undergoing clinical trials (*19*). The effect may result from molecular mechanisms such as the

ABBREVIATIONS: RT = radiation therapy; ER = enhancement ratio(s); PLDR = potentially lethal damage recovery; TGF = therapeutic gain factor.

---

[1] Supported in part by Public Health Service grant CA-40500 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and by Johnson Matthey, Inc.

[2] Radiobiology Laboratories, Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Hanover, NH.

* *Reprint requests:* Evan B. Douple, Ph.D., Radiobiology Laboratories, Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Hanover, NH 03756.

inhibition of repair of radiation damage by the Pt complexes, since it appears to result 1) from reduced recovery from RT-induced potentially lethal damage when cisplatin is present during the post-RT incubation of plateau-phase cells and 2) from reduced recovery from RT-induced sublethal damage when cisplatin is present between two split courses of RT. Several generalizations can be made regarding these effects.

Platinum Administered Before Radiation

The following are generalizations regarding administration of Pt to cells before RT:

1) A small amount of radiosensitization has been reported by several investigators (*4,5,17,20*) using a variety of mammalian cell lines and different concentrations of cisplatin (approximately 10 μM) that produce significant chemotoxicity.

2) In some laboratories (*4,20*) and in certain cell lines, this radiosensitization by cisplatin produces ER only under hypoxic conditions during RT, while in other laboratories (*5,17*), it produces ER under well-oxygenated conditions during RT.

3) When cisplatin was combined with misonidazole prior to RT, ER values were higher than expected from the additive effects of the two radiosensitizers (*20*).

4) Several Pt analogs, including the *trans*-isomer of the parent cisplatin and second-generation chemotherapy complexes such as paraplatin and iproplatin, have been shown to produce radiosensitization (*4,6–15,19–22*). If the interaction with RT is dependent on the number of Pt atoms present in the cells, then Pt complexes that are less cytotoxic may permit higher levels of Pt in cells, with the potential for a more significant potentiation of RT-induced cell killing.

5) The radiosensitizing effect is not unique to Pt complexes since complexes of other metals have produced this effect in mammalian cells (*23,24*), and it does not require attendant chemotoxicity for all complexes since exposure to certain nontoxic metal complexes has produced the effect.

6) While free radical chemistry may play a role in this phenomenon, the effects are undoubtedly complex and may involve additional mechanisms such as sulfhydryl depletion, radiolytic toxic product formation, and cell-cycle perturbations. It is not clear whether an association of the Pt complexes with DNA, such as that in adduct formation, results in enhancement of this interaction with RT.

Platinum Administered After Radiation

The following are generalizations regarding administration of Pt to cells after RT:

1) Potentiation of cell killing by Pt after RT has been reported for doses of Pt complexes lower than those required for hypoxic cell radiosensitization (*16–19*).

2) Several Pt analogs, including the *trans*-isomer of the parent cisplatin and the second-generation chemotherapy complexes paraplatin and iproplatin (*19*), have been shown to produce this effect.

3) The effect does not require attendant chemotoxicity for all complexes.

4) The enhanced cell killing might result from mechanisms other than inhibition of repair. For example, an ethylenimine–Pt complex has produced survival levels below those predicted for total inhibition of PLDR, and this effect has been termed an enhanced chemotoxicity, suggesting that irradiated cells might become more sensitive to Pt chemotherapy after RT (*18*).

5) It is not clear whether an association of the Pt complexes with DNA, such as that in adduct formation, results in enhancement of this interaction with RT.

INTERACTIONS IN ANIMAL STUDIES

Several studies have demonstrated that cisplatin or other Pt analogs produce an enhanced therapeutic effect on transplanted tumors when combined with RT (*2,7–13, 21,25–30*). In these animal tumor systems, these agents have antitumor activity such that it is frequently difficult to prove that the results are supra-additive, i.e., greater than the additive effect of either agent acting alone. The enhanced effects on tumors approximate those reported using misonidazole as a radiosensitizer and have frequently not been accompanied by an equivalent enhancement of normal tissue damage. Whether this apparent therapeutic gain will be realized in clinical trials remains to be demonstrated. While it is hoped that pharmacokinetic, mechanistic, or time-sequencing studies will suggest a clear rationale predicting therapeutic gain, some evidence for potentiation of damage in normal intestine and skin warns that one must extrapolate from the animal data to the clinical protocol with caution.

The delivery of intratumoral Pt using an associated Pt matrix, as reported in this meeting (*27,28*), may represent another method for increasing the concentration of Pt compounds for subsequent interaction with RT. Total Pt levels measured in transplanted murine mammary tumors following cisplatin or paraplatin ip approach the levels required for in vitro radiosensitization or post-RT potentiation (i.e., >10 μM) (*31*). In this study, the Pt concentration was computed by measuring the total Pt per gram of tumor and assuming that 1 g of tumor is equivalent to 1 ml of solvent.

The following are generalizations from Pt-RT studies in animals:

1) Evidence for potentiation of RT by cisplatin has been demonstrated in several animal tumor systems in several laboratories (*2,7–13*). For example, an ER value of 1.7 was reported for single doses of Pt combined with RT, using tumor cure as an assay end point in a mouse tumor system (*25*).

2) Greater effects appear to result when multiple fractions of Pt and RT are combined, such as the ER of 1.9 reported for a study (*29*) in which cisplatin was one of only two chemotherapeutic drugs to produce supra-additivity when coordinated with RT in certain protocols.

3) As a general rule, most studies have reported that normal skin did not show a significant potentiation of damage or that the enhancement in normal tissues was less than that produced in the tumor (*30*).

4) There have been reports of some potentiation of damage to skin (*32,33*); intestinal crypt cells (*34,35*) [including inhibition of RT-induced repair by cisplatin (*36,37*)]; and mouse esophagus (*38*).

5) The potentiation of effects on tumor cells has also been demonstrated using low-dose-rate RT with concurrent infusion of cisplatin (*26*).

6) Higher doses of some of the second-generation Pt complexes relative to cisplatin are tolerated by mice, and it is not clear whether these higher levels produce greater therapeutic enhancement in all animal tumor systems.

RESULTS OF CLINICAL STUDIES

The encouraging results of preclinical studies have led to several ongoing pilot clinical trials, which have combined cisplatin with RT in an attempt to exploit interactions between Pt and RT in a variety of tumor types (*10,11,39*). There are two operationally defined effects observed in vitro and presumably operating in vivo that might produce an improved TGF: radiosensitization of hypoxic cells and post-RT potentiation such as inhibition of PLDR. Radiosensitization of hypoxic cells has been a major goal of radiobiology for three decades, and radiation oncology is awaiting results of clinical testing of the latest generation of electron-affinic radiosensitizers. If hypoxic cells are restricted to tumor tissue, then it is apparent how an ER in these radioresistant cell populations should favor a TGF. Even if radiosensitization of well-oxygenated cells occurs, it might produce a selective enhanced cell kill in tumors if metal complexes are targeted to tumor cells or if RT is delivered when concentrations of the active metal complex in tumors are significantly higher than those in normal cells.

Inhibition of PLDR might produce a TGF if this recovery is characteristic of tumor cells rather than normal cells, as has been proposed by Weichselbaum et al. (*40*), but a role of PLDR in clinical tumors is speculative at this time. In Japan, clinical trials are currently employing PLDR inhibitors in an attempt to evaluate the effectiveness of this approach to cancer therapy.

Most clinical studies reported to date have accrued relatively small numbers of patients and have attempted to assess feasibility rather than to demonstrate improved efficacy. The delay in the development of clinical protocols since the earliest reports of Pt-RT interactions has probably been due to several factors, including the following:

1) The effects in cultured mammalian cells have been rather modest compared to those for misonidazole and RT, and a variety of responses have been reported to be dependent on drug dose, scheduling, experimental system, cell line, and laboratory.

2) Although the doses required to demonstrate the interactions are quite low compared to those for misonidazole, they are limited experimentally by chemotoxicity. The Pt levels required for interaction with RT might exceed the limits attained in clinical solid tumors throughout most of the RT fractionation, using current Pt complexes and protocols.

3) The cytotoxicity of cisplatin and other chemotherapeutic complexes complicates the interpretation of experimental results in a climate that has been biased to restrict radiosensitizers to agents that are not active when used alone; furthermore, no single mechanism has been described to explain the interaction under a multitude of conditions.

4) The nephrotoxicity and emesis produced by cisplatin is significant, and the drug has a bad reputation for other toxic effects and for morbidity.

5) Approaches to combining chemotherapeutic agents with RT have been conservative and cautious since experience with other agents has demonstrated that the combined modality approach frequently leads to an enhancement of normal tissue damage and alters the known patterns of response to RT.

6) There has been a lack of pharmacologic data as well as a lack of information regarding the importance of timing in the administration of Pt and RT. It is clear that coordination of the administration of the two agents may place serious constraints on the scheduling of the Pt chemotherapy and RT.

Results of phase III randomized clinical trials are not yet available. However, it would be very fortuitous if the current experimental protocols represent the optimal time, dose, and sequence relationships for this combined modality approach to cancer therapy.

The appropriate active chemical form of the Pt complex required for the interaction is not known, and the optimal concentrations of Pt in solid human tumors as a function of time after administration are not well-known. In pharmacokinetic studies of Pt levels using atomic absorption spectrometry after iv infusion of cisplatin, it has been shown that plasma levels have short half-lives (approximately 30 min) (*41*). In addition, levels of Pt representing presumably bound Pt in tumor tissue have been measured at long time periods of several days. The levels of Pt per gram of wet tissue measured in squamous cell carcinoma patients (*42*) begin to approach the concentration of cisplatin required to produce Pt-RT interactions in cultured mammalian cells (10 μM).

Since second and future generations of Pt complexes are expected to play a key role in cancer chemotherapy, it is important to consider that certain of these metal complexes might prove to be more interactive with RT than cisplatin. It is also reasonable to expect that clinically acceptable metal complexes might be specifically designed and synthesized to serve as potentiators of RT. The potential for the design of such metal radiosensitizers has been illustrated by in vitro studies of FLAP, a Pt complex with two metronidazole moieties attached to dichloroplatinum (II) (*43*), and 2-NIPt, a Pt complex that incorporates two electron-affinic azomycin moieties (*44*). In addition, there is interest in the hypothesis that depletion of glutathione and other sulfhydryls in patients may significantly enhance the interactions of cisplatin or other metal complexes with RT. Methods for the delivery of higher systemic doses of cisplatin have been introduced clinically, and it is not known whether these protocols will be more appropriate for combination with RT.

CONCLUSIONS

A decade has passed since Richmond and Powers (*3*) reported that cisplatin was a potent radiosensitizer of hypoxic bacteria and since a small amount of radiosensitization was observed in mammalian cells and in animal tumors. A post-RT potentiation effect, which is operationally attributed to inhibition of recovery from potentially lethal damage and sublethal damage, has also become part of a rationale for combining Pt with RT. It is fortuitous that in a period of time when radiobiologic investigations have placed a major focus on the development of hypoxic cell radiosensitizers and when an interest in inhibitors of

PLDR is revived, a parent drug representing a new class of chemotherapeutic agents is introduced into the clinic, and that this drug, cisplatin, happens to produce both of these modes of potentiation when combined with RT. However, the mechanisms responsible for the observed Pt-RT interactions remain unknown, and preclinical studies have reported quite variable effects. Additional preclinical mechanistic studies are required. Furthermore, no results from a phase III randomized clinical trial have been reported to date to suggest improved efficacy and therapeutic gain. It is possible that, if results of clinical trials are favorable, potentiation and therapeutic gain could be enhanced by optimal protocol designs based on improved understanding of Pt-RT mechanisms and of the incorporation of new metal complexes, including drugs designed to exploit the interactions.

REFERENCES

(*1*) Zak M, Drobnik J: Effects of *cis*-dichlorodiammineplatinum(II) on the post-irradiation lethality in mice after irradiation with X-rays. Strahlentherapie 142:112–115, 1971.

(*2*) Wodinsky I, Swiniarski J, Kensler CJ, et al: Combination radiotherapy and chemotherapy for P388 lymphocytic leukemia in vivo. Cancer Treat Rep 4:73–80, 1974.

(*3*) Richmond RC, Powers EL: Radiation sensitization of bacterial spores by *cis*-dichlorodiammineplatinum(II). Radiat Res 68:20–23, 1976.

(*4*) Douple EB, Richmond RC: Platinum complexes as radiosensitizers of hypoxic mammalian cells. Br J Cancer 37:98–102, 1978.

(*5*) Alvarez MV, Cobreros G, Heras A, et al: Studies on *cis*-dichlorodiammineplatinum(II) as a radiosensitizer. Br J Cancer 37:68–72, 1978.

(*6*) Szumiel I, Nias AHW: The effect of combined treatment with a platinum complex and ionizing radiation on Chinese hamster ovary cells in vitro. Br J Cancer 33:450–458, 1976.

(*7*) Douple EB, Richmond RC: Radiosensitization of hypoxic tumor cells by *cis*- and *trans*-dichlorodiammineplatinum(II). Int J Radiat Oncol Biol Phys 5:1369–1372, 1979.

(*8*) Douple EB, Richmond RC: A review of platinum-complex biochemistry suggests a rationale for combined platinum-radiotherapy. Int J Radiat Oncol Biol Phys 5:1335–1339, 1979.

(*9*) Douple EB, Richmond RC: Interactions between platinum coordination complexes and ionizing radiation. *In* Cisplatin: Current Status and New Developments (Prestayko AW, Crooke ST, Carter SK, eds). New York: Academic Press, 1980, pp 125–147.

(*10*) Douple EB: The use of platinum chemotherapy to potentiate radiotherapy: Preclinical results encourage clinical trials. Platinum Metals Rev 29:118–125, 1985.

(*11*) Douple EB, Richmond RC, O'Hara JA, et al: Carboplatin as a potentiator of radiation therapy. Cancer Treat Rev 12(suppl A):111–124, 1985.

(*12*) Nias AHW: Radiation and platinum drug interaction. Int J Radiat Biol 48:297–314, 1985.

(*13*) Douple EB: *cis*-Diamminedichloroplatinum(II): Effects of a representative metal coordination complex on mammalian cells. *In* Cell Cycle Effects of Drugs (Dethlefsen LA, ed). Oxford, England: Pergamon Press, 1986, pp 215–250.

(*14*) Richmond RC: Toxic variability and radiation sensitization by dichlorodiammineplatinum (II) complexes in *Salmonella typhimurium* cells. Radiat Res 99:596–608, 1984.

(*15*) Richmond RC, Khokhar AR, Teicher BA, et al: Toxic variability and radiation sensitization by Pt (II) analogs in *Salmonella typhimurium* cells. Radiat Res 99:609–626, 1984.

(*16*) Dritschilo A, Piro AJ, Kellman AD: The effect of *cis*-platinum on the repair of radiation damage in plateau phase Chinese hamster (V79) cells. Int J Radiat Oncol Biol Phys 5:1345–1349, 1979.

(*17*) Carde P, Laval F: Effects of *cis*-dichlorodiammine platinum II and X-rays on mammalian cell survival. Int J Radiat Oncol Biol Phys 7:929–933, 1981.

(*18*) Lindquist K, Douple EB, Richmond RC: (Abstr) Platinum drug modulation of radiation damage. Radiat Res 91:408, 1982.

(*19*) O'Hara JA, Douple EB, Richmond RC: Enhancement of radiation-induced cell kill by platinum complexes (carboplatin and iproplatin) in V79 cells. Int J Radiat Oncol Biol Phys 12:1419–1422, 1986.

(*20*) Stratford IJ, Williamson C, Adams GE: Combination studies with misonidazole and a *cis*-platinum complex: Cytotoxicity and radiosensitization in vitro. Br J Cancer 41:517–522, 1980.

(*21*) Teicher BA, Holden SA: Antitumor and radiosensitizing activity of several platinum-(+) dye complexes. Radiat Res 109:58–67, 1987.

(*22*) Teicher BA, Rockwell S, Lee JB: Radiosensitization of EMT6 cells by four platinum complexes. Int J Radiat Oncol Biol Phys 11:937–941, 1985.

(*23*) Teicher BA, Jacobs JL, Cathcart KNS, et al: Some complexes of cobalt (III) and iron (III) are radiosensitizers of hypoxic EMT6 cells. Radiat Res 109:36–46, 1987.

(*24*) Douple EB, Green CJ, Simic MG: Potentiation of cellular radiosensitivity by nitroprusside and vitamin B_{12}. Int J Radiat Oncol Biol Phys 6:1545–1549, 1980.

(*25*) Overgaard J, Khan AR: Selective enhancement of radiation response in a C3H mammary carcinoma by cisplatin. Cancer Treat Rep 65:501–503, 1981.

(*26*) Fu KK, Rayner PA, Lam KN: Modification of the effects of continuous low dose rate irradiation by concurrent chemotherapy infusion. Int J Radiat Oncol Biol Phys 10:1473–1478, 1984.

(*27*) Begg AC, Bartelink H, Stewart FA, et al: Improvement of differential toxicity between tumor and normal tissues using intratumoral injection with or without a slow-drug-release matrix system. NCI Monogr 6:133–136, 1988.

(*28*) Howes AE, Herman TS, Montoya VP, et al: Effects of matrix-associated chemotherapy in combination with irradiation in vivo. NCI Monogr 6:141–143, 1988.

(*29*) Lelieveld P, Scoles MA, Brown JM, et al: The effect of treatment in fractionated schedules with the combination of X-irradiation and six cytostatic drugs on the RIF-1 tumor and normal mouse skin. Int J Radiat Oncol Biol Phys 11:111–121, 1985.

(*30*) Bartelink H, Kallman RF, Rapacchietta D, et al: Therapeutic enhancement in mice by clinically relevant dose and fractionation schedules of *cis*-diamminedichloroplatinum(II) and irradiation. Radiother Oncol. In press.

(*31*) Douple EB, Totten MD, Spencer F, et al: Platinum levels in murine tumor following intraperitoneal administration of cisplatin or paraplatin. NCI Monogr 6:129–132, 1988.

(*32*) Douple EB, Eaton WL, Tulloh ME: Skin radiosensitization studies using combined *cis*-dichlorodiammineplatinum(II) and radiation. Int J Radiat Oncol Biol Phys 5:1383–1385, 1979.

(*33*) von der Maase H: Effect of cancer chemotherapeutic drugs on the radiation-induced skin reactions in mouse feet. Br J Radiol 57:697–707, 1984.

(*34*) Schenken LL, Burholt DR, Hagemann RF, et al: Combined modality oncotherapies: Cell kinetic approaches for avoidance of gastrointestinal toxicity. *In* Frontiers of

Radiation Therapy and Oncology (Vaeth JM, ed). Basel, Switzerland: S Karger, 1979, pp 82–101.

(*35*) PHILLIPS TL: Clinical and experimental alterations in the radiation therapeutic ratio caused by cytotoxic chemotherapy. *In* Radiation Biology Cancer Research (Meyn RE, Withers HR, eds). New York: Raven Press, 1980, pp 567–588.

(*36*) LUK KH, ROSS GY, PHILLIPS TL, et al: The interaction of radiation and *cis*-diamminedichloroplatinum(II) in intestinal crypt cells. Int J Radiat Oncol Biol Phys 5:1417–1420, 1979.

(*37*) BURHOLT DR, SCHENKEN LL, KOVACS CJ, et al: Response of the murine gastrointestinal epithelium to *cis*-dichlorodiammineplatinum II. Radiation combinations. Int J Radiat Oncol Biol Phys 5:1377–1381, 1979.

(*38*) PHILLIPS TL: Rationale for the selection of combined treatment schedules using fractionated radiation and chemotherapy. *In* Advances in Medical Oncology, Research and Education (Moore M, ed), vol VI. Elmsford, NY: Pergamon Press, 1979, pp 183–190.

(*39*) COUGHLIN CT, RICHMOND RC: Platinum based combined modality approach for locally advanced head and neck carcinoma. Int J Radiat Oncol Biol Phys 11:915–919, 1985.

(*40*) WEICHSELBAUM RR, SCHMIT A, LITTLE JB: Cellular repair factors influencing radiocurability of human malignant tumors. Br J Cancer 45:10–16, 1982.

(*41*) STERNSON LA, REPTA AJ, SHIH H, et al: Disposition of cisplatin vs total platinum in animals and man. *In* Platinum Coordination Complexes in Cancer Chemotherapy (Hacker MP, Douple EB, Krakoff IH, eds). Boston: Martinus Nijhoff, 1984, pp 126–137.

(*42*) MATTOX DE, STERNSON LA, VON HOFF DD, et al: Tumor concentration of platinum in patients with head and neck cancer. Otolaryngol Head Neck Surg 91:271–275, 1983.

(*43*) BALES JR, SADLER PJ, COULSON CJ, et al: Hypoxic cell sensitization to radiation damage by a new radiosensitizer: *cis*-dichloro-bis(1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole-N3) platinum(II) (FLAP). Br J Cancer 46:701–705, 1982.

(*44*) DOUPLE EB, TEICHER BA: Potentiation of cell kill by combining nitroimidazole-platinum complexes with radiation. *In* Platinum Coordination Complexes in Cancer Chemotherapy (Hacker MP, Douple EB, Krakoff IH, eds). Boston: Martinus Nijhoff, 1984, p 355.

Combined Modality Treatment of Peritoneal Mesotheliomas

Gilbert S. Lederman,[1,*] Abram Recht,[1] Terence Herman,[2] Robert Osteen,[3] Joseph Corson,[4] and Karen H. Antman[2]

ABSTRACT—**Five patients with peritoneal mesothelioma were treated with a multimodality approach consisting of debulking surgery, ip chemotherapy, and whole-abdomen irradiation. Chemotherapy included cisplatin and doxorubicin; radiation was given by an open-field technique. Three patients remain disease free at 46, 60, and 61 months after diagnosis. This treatment approach may serve as a model for common tumors characterized by abdominal carcinomatosis.—NCI Monogr 6:321–322, 1988.**

Aggressive local therapy of peritoneal mesotheliomas offers an attractive approach to a disease that remains confined to the abdominal cavity for most of its course (*1*). This report presents an analysis of the results in 5 patients who received treatment with curative intent consisting of whole-abdomen irradiation, ip cisplatin plus doxorubicin chemotherapy, and debulking surgery. The success of this approach is important in view of the previously bleak outcome of this disease.

PATIENTS AND METHODS

Five patients with diffuse peritoneal mesothelioma included 3 males aged 27, 39, and 40 years and 2 females aged 31 and 34 years (table 1). The median ip dose of cisplatin was 75 mg/m^2, with a range of 20–100 mg/m^2. The median ip dose of doxorubicin was 35 mg, with a range of 20–50 mg.

Two patients received iv prior to ip chemotherapy. Patient #4 received mitomycin and cisplatin iv, whereas patient #5 received cyclophosphamide and doxorubicin iv and radiation prior to ip treatment. One patient subsequently underwent successful surgical debulking and proceeded with the other treatment modalities. One patient received ip treatment because his blood counts fell after earlier therapy. Open-field radiotherapy was given after the completion of ip chemotherapy to 4 of the 5, and before drug administration to the fifth patient. Dose to the abdominal field was 3,000 cGy. Fraction sizes ranged from 100 to 180 cGy; however, 2 patients who were treated twice daily received 100–150 cGy. Two patients had pelvic boosts to a total dose of approximately 4,500 cGy. Full-thickness kidney blocks were added at 1,400–1,800 cGy and partial liver blocks at 2,000–2,700 cGy. All patients had at least 3 laparotomies for diagnosis or debulking. Details of treatment are given in tables 1 and 2.

Survival is measured from the date on which a pathologic diagnosis was made. No patient was lost to follow-up. Patients are followed with physical examinations, screening blood tests (blood counts, liver functions), chest radiographs, and abdominal-pelvic computerized tomography.

RESULTS

Three patients remain disease free at 46, 60, and 61 months after diagnosis (4 yr after completing treatment). Toxicity included nausea and vomiting and transient pancytopenia. One patient is receiving intermittent iv hyperalimentation because of chronic malabsorption.

DISCUSSION

The uniform death of most untreated patients with peritoneal mesothelioma has been well documented.

The disease-free survival of 46–61 months in 3 patients selected to be treated with surgical debulking, chemotherapy, and whole-abdomen irradiation is remarkable. In a disease known for its progressive course and fatal outcome, this pilot study suggests that an aggressive interdisciplinary approach may improve prognosis (*2–4*).

Debulking surgery (like that used in ovarian carcinoma) leaves small tumor implants (*5,6*). These remaining implants may respond to local therapy better than would gross disease. The major advantage of ip chemotherapy is that the patient achieves a local concentration of cytotoxic agent at least a log higher than that attained with iv administration (*6,7*). Systemic distribution after ip administration likely adds to the efficacy of these drugs by delivery of the agent via capillary blood. The second-look laparotomies in this series have confirmed the response of the mesothelioma to cisplatin and doxorubicin chemotherapy and allow further resection of residual disease.

Although the benefit observed here is based upon a few patients, the success to date of this multimodality approach makes a compelling argument for further investigation. Clinical studies with long-term follow-up will be needed to answer the question if this previously fatal disease is now potentially curable.

---

[1] Joint Center for Radiation Therapy and Department of Radiation Therapy, Harvard Medical School, Boston, MA.

[2] Department of Medical Oncology, Dana-Farber Cancer Institute, and Department of Medicine, Harvard Medical School, Boston, MA.

[3] Department of Surgery, Brigham and Women's Hospital, and Department of Surgery, Harvard Medical School, Boston, MA.

[4] Department of Pathology, Brigham and Women's Hospital, and Department of Pathology, Harvard Medical School, Boston, MA.

[*] *Reprint requests:* Gilbert S. Lederman, M.D., Department of Radiation Oncology, Staten Island Hospital, 475 Seaview Ave., Staten Island, NY 10305.

Table 1.—Treatment regimen of 5 patients with peritoneal mesothelioma[a]

				XRT, cGy					
				Upper abdomen			Pelvis		
Patient No.	Age, yr	Sex	Sequence of treatment	Total dose	Fraction size	No. of days	Total dose	Fraction size	No. of days
1	31	Female	Lap (bx) > Lap (deb) > ip > Lap (deb) > XRT	3,075	150	36	4,515	180	54
2	34	Female	Lap (deb) > Lap (deb) > ip > Lap (exploratory) > Lap (deb) > XRT	3,000	150	35	3,000	150	35
3	39	Male	Lap (bx) > Lap (deb) > ip > Lap (deb) > XRT	3,000	100[b]	25	3,000	100	25
4	27	Male	Lap (bx) > iv > Lap (deb) > ip > Lap (deb) > XRT	3,000	150	35	3,000	150	35
5	40	Male	Lap (deb) > iv > XRT > Lap (deb) > ip > Lap (catheter removal)	3,039	100[b]	25	4,560	150[b]	45

[a] Lap = laparotomy; bx = biopsy; deb = debulking surgery; XRT = radiotherapy; > = followed by.

[b] Treatment was given twice daily.

Table 2.—Chemotherapy cycles and interval since diagnosis

Patient No.	Disease-free interval since diagnosis, mo	No. of ip drug cycles	
		Doxorubicin	Cisplatin
1	46+	5	5
2	60+	6	3
3	21	4	4
4	38	9	3
5	61+	3	6

REFERENCES

(*1*) Kannerstein M, Churg J: Peritoneal mesothelioma. Hum Pathol 8:83–94, 1977.

(*2*) Antman KH, Osteen RT, Klegar KL, et al: Early peritoneal mesothelioma. A treatable malignancy. Lancet 1:977–981, 1985.

(*3*) Antman KH, Pomfret EA, Aisner J, et al: Peritoneal mesothelioma: Natural history and response to chemotherapy. J Clin Oncol 1:386–391, 1983.

(*4*) Lederman GS, Recht A, Herman T, et al: Long-term survival in peritoneal mesothelioma: The role of radiotherapy and combined modality treatment. Cancer 59:1882–1886, 1987.

(*5*) Cohen CJ: Surgical considerations in ovarian cancer. Semin Oncol 12:53–56, 1985.

(*6*) Griffiths CT, Grogran RH, Hall TC: Advanced ovarian cancer: Primary treatment with surgery, radiotherapy and chemotherapy. Cancer 29:107–111, 1972.

(*7*) Markman M, Cleary S, Lucas WE, et al: Intraperitoneal chemotherapy with high-dose cisplatin and cytosine arabinoside for refractory ovarian carcinoma and other malignancies principally involving the peritoneal cavity. J Clin Oncol 3:925–931, 1985.

Continuous Infusion of Etoposide, Bolus Administration of Cisplatin, and Simultaneous Radiation Therapy in Previously Treated Patients With Small Cell Bronchogenic Carcinoma

Kendrith M. Rowland, Jr.,[1,*] Philip Bonomi,[2] Samuel G. Taylor IV,[2] Susan Maffey,[3] Salitha Reddy,[2] and Myung-Sook Lee[2]

ABSTRACT—**Thirty-one patients with previously treated small cell bronchogenic carcinoma were treated with split-course radiation therapy and concurrent cisplatin and etoposide. Twenty-four patients (78%) had clinical partial or complete responses. Median survival from the time of salvage was 6.3 months. Fifty-eight percent of the patients failed on therapy initially in sites outside the radiation port. Hematologic toxicity was moderate. Concurrent cisplatin, etoposide, and split-course radiation therapy is feasible and active in the treatment of small cell bronchogenic carcinoma.—NCI Monogr 6:323-325, 1988.**

Median survival from the time of relapse from initial therapy for SCBC is reported to be 7 to 8 weeks (*1*). Recently, cisplatin and etoposide in combination have been noted to have significant activity in previously treated patients with SCBC (*2-5*). Response rate for the combination, 12%-55%, generally appears to be higher than that observed for cisplatin and etoposide as single agents (*1*). Median survival from the initiation of this combination therapy has ranged from 12 to 35 weeks. In addition, radiation therapy alone for refractory disease in the chest has a response rate of 64% and a median survival of 15 weeks (*6*). The role of cisplatin as a radiation sensitizer is well established (*7*); that of etoposide is suggested, although not as yet fully evaluated (*8*). The schema of concurrent cisplatin, etoposide, and split-course radiation therapy was developed in our attempt to maximize their possible synergistic and radiosensitizing properties.

As the schedule of etoposide appears optimal in in vitro studies with continuous exposure (*9*) and in patients with SCBC with frequent dosing, i.e., daily versus weekly (*10*), it was administered as a continuous venous infusion.

Split-course radiation therapy was delivered to sites of symptomatic recurrent or persistent disease in the chest or liver. This report is an update of a previous report on the initial 15 patients treated with this regimen (*11*).

PATIENTS AND METHODS

Patients with histologically or cytologically confirmed SCBC refractory to previous chemotherapy were evaluated. Eligibility requirements included measurable disease; failure or progression on one or more chemotherapy regimens; Eastern Cooperative Oncology Group performance status 0, 1, 2, 3; and adequate hematologic, renal, and biliary function (WBC count over 4,000/mm^3, platelet count over 100,000/mm^3, BUN less than 25 mg/dl, creatinine under 1.5 mg/dl, and bilirubin under 2 mg/dl). All patients underwent computerized tomography scans of the chest and upper abdomen and bone scans prior to initiating retreatment.

Concomitant daily radiation therapy and a continuous infusion of etoposide were given on days 1 through 5. Cisplatin was given on day 1. The initial 13 patients received 200 cGy radiation therapy once daily; the remaining 18 patients were given 116 cGy twice daily. Each 5-day cycle of chemotherapy and radiation therapy was scheduled initially every 2 weeks; however, later this was changed to every 3 weeks for the majority of the patients. All patients began at 60 mg etoposide and cisplatin$\cdot$m$^{-2}\cdot$day^{-1}, except those with performance status 3 for which initial doses were 50% lower. Dose modifications were made on the basis of hematologic and renal values on day 1. If the WBC count was less than 2,000/mm^3, platelet count less than 75,000/mm^3, or creatinine over 2.5 mg/dl, treatment was delayed 1 week. Involved field radiation to one symptomatic or persistent site was administered with simultaneous chemotherapy for up to 4 cycles. Thereafter, patients received either 1 or 2 more combined modality therapy cycles with shrinking radiation fields, maintenance chemotherapy, or observation at investigator discretion. Standard Eastern Cooperative Oncology Group criteria were used in the evaluation of progression and response.

RESULTS

Thirty-one patients entered on study could be evaluated for response; all but 1 has died. Performance status, extent of disease at re-treatment, and number of prior treatment regimens are listed in table 1. Prior treatment consisted of CAV in 14 patients, CCM/AVP-16 in 6, and a variety of other combinations (number of drugs 4 to 6) in 11. Eleven patients received etoposide previously. Twenty-four (77%)

ABBREVIATIONS: SCBC = small cell bronchogenic carcinoma; CAV = cyclophosphamide, doxorubicin, vincristine; CCM/AVP-16 = cyclophosphamide, doxorubicin, lomustine, methotrexate, and etoposide.

---

[1] Division of Medical Oncology, Carle Clinic Association, Urbana, IL.

[2] Department of Medicine, Section of Medical Oncology (*P. Bonomi* and *S. G. Taylor IV*) and Department of Radiation Therapy (*S. Reddy* and *M-S. Lee*), Rush Medical College, Chicago, IL.

[3] Radiation Oncology Center, East Carolina University School of Medicine, Greenville, NC.

[*] *Reprint requests:* Kendrith M. Rowland, Jr., M.D., Carle Clinic Association, 602 W. University, Urbana, IL 61801.

Table 1.—Patient characteristics

Median age, yr	60
Male:female ratio	19:12
Performance status	
0, 1	18
2	11
3	2
Limited disease (at re-treatment)	17
Extensive disease (at re-treatment)	14
Previous chemotherapy regimens	
1	23
2	8

patients failed on therapy. Median time on chemotherapy prior to relapse was 6 months (range 1–12).

Radiation therapy was administered to the chest to 25 and to the liver to 6 patients. Of all patients, only the 6 receiving liver radiation had received prior chest radiation. All patients received a minimum of 2 cycles of combined modality therapy (median 4). Eight patients were given 1 to 5 (median 2) cycles of maintenance etoposide and cisplatin chemotherapy. Median radiation doses to the liver and chest were 1,600 and 4,000 cGy, respectively. One hundred six combined modality treatment cycles were given, 25 (24%) of which were delayed beyond a 2-week rest period. Doses were reduced to 30 mg/m^2 for cisplatin and etoposide in 30% and 36% of the 106 cycles, respectively. The percents (actual/full dose) of cisplatin and etoposide were 79 and 77, respectively.

Of 31 patients, 7 (23%) achieved a clinical complete response and 17 (55%) a partial response. Response rate did not differ statistically in patients receiving prior CAV, CCM/AVP-16, other regimens, or those with a treatment-free relapse. Response to salvage therapy did not correlate with response to initial treatment or time to failure. Seven of 11 (64%) patients with prior exposure to etoposide responded. Five of the 6 patients receiving liver radiation had partial remission.

Survival from the time of diagnosis and salvage treatment with cisplatin, etoposide, and split-course radiation was 15.6 and 6.3 months, respectively. Overall, 18 of 31 patients (58%) failed outside of the radiation field, 10 (32%) within, and 3 (10%) both in and outside of the field.

Toxicity was primarily hematologic. Of the WBC and platelet counts evaluated every 2–3 weeks throughout treatment, the median lowest counts were 2,100/mm^3 and 150,000/mm^3, respectively. Only 1 patient had a transient rise in creatinine above 2 mg/dl. Esophagitis was moderate in 1 patient. All patients were hospitalized during the 5-day treatment cycles.

DISCUSSION

Cisplatin and etoposide as salvage therapy for SCBC has most often been administered with the latter drug given as an iv bolus and without concurrent radiation therapy. Patient characteristics and results of combination drug trials reported in some detail are summarized in table 2.

Evans and co-workers (*2*) noted a 55% response rate in a large number of patients failing CAV chemotherapy. Median survival from initiation of cisplatin and etoposide was 8.7 and 5.2 months for patients with limited and extensive disease, respectively. Overall survival was not noted; however, less than 15% of these patients were alive at 16 months. One explanation for this perhaps lower than expected 16-month survival might be the delay (or 6-mo median treatment-free interval) of the perhaps more effective cisplatin and etoposide therapy.

A higher dose of chemotherapy, i.e., 40 mg cisplatin/m^2 and 200 mg etoposide/m^2 on days 1–3, was used in 29 patients previously treated with a median of 4 drugs by Porter and associates (*3*). Response rate was 52%. Interestingly, 60% of the 15 patients who received previous etoposide responded. Overall survival was not reported.

Lopez et al. (*4*) also treated 30 patients with cisplatin and etoposide, all of whom had failed initial 3- or 4-drug therapy. Although overall response rate to therapy was 27%, the response rate in 12 patients with a performance status of 1–2 was 50%.

Combination cisplatin and etoposide therapy was least active in a group of 29 patients studied by Batist and co-workers (*5*). The low response rate of 12% was attributed primarily to suspected cellular resistance from previous multidrug therapy. All but 1 patient failed on a 6-drug regimen of cyclophosphamide, lomustine, methotrexate, vincristine, doxorubicin, and procarbazine. Overall median survival for the group was not reported.

In the present study, with etoposide given as an infusion

Table 2.—Cisplatin and VP-16 in previously treated patients with SCBC[a]

					Initial treatment					
							Patients receiving ≥ 5 drugs			
Investigators	References	No. of patients	Performance status 0, 1, %	Response rate, %	Median cycles or months of chemotherapy	Patients receiving chest RT, %	No.	%	MST from diagnosis, mo	MST from cisplatin/etoposide therapy, mo
Evans et al.	(*2*)	78	60	55	6 cycles	71	5	6	NR	8.6[b] 5.2[c]
Lopez et al.	(*4*)	30	40	27	NR	40	0	0	13	4
Porter et al.	(*3*)	29	NR	52	5 cycles	NR	NR		NR	4
Batist et al.	(*5*)	29	62	12	7 mo	83	28	97	NR	3
Rowland et al. (this study)		31	55	78	6 mo	13	12	39	15.6	6.3

[a] RT = radiation therapy; MST = median survival time; NR = not reported.

[b] Value is for patients with limited disease.

[c] Value is for patients with extensive disease.

and the addition of concurrent radiation therapy, the response rate was 78%. No correlation among the response rate and number of previous drugs received, presence of a treatment-free interval, or previous exposure to etoposide was observed. In 6 patients failing the CCM/AVP-16 regimen, differing only from the addition of etoposide and absence of procarbazine and vincristine as used by Batist, the response rate was 66%. Whether the addition of radiation therapy overcame multidrug resistance is unknown. Benefit from infusion versus bolus etoposide has not been demonstrated clinically; however, infusion with simultaneous cisplatin has not been evaluated systematically.

Overall, the median survival of 15.6 months and 78% response rate in the present study involving a heavily pretreated population are perhaps higher than might be expected. The scheduling of cisplatin and etoposide and split-course radiation as used in this study might prove beneficial in the initial treatment of patients with SCBC.

REFERENCES

(*1*) Morstyn G, Ihde DC, Lichter AS, et al: Small cell lung cancer 1973–1983: Early progress and recent obstacles. Int J Radiat Oncol Biol Phys 10:515–539, 1984.

(*2*) Evans WK, Osoba D, Feld R, et al: Etoposide and cisplatin: An effective treatment for relapse in small cell lung cancer. J Clin Oncol 3:65–71, 1985.

(*3*) Porter LL III, Johnson DH, Hainsworth JD, et al: Cisplatin and etoposide combination chemotherapy for refractory small cell carcinoma of the lung. Cancer Treat Rep 69:479–481, 1985.

(*4*) Lopez JA, Mann J, Grapski RT, et al: Etoposide and cisplatin salvage chemotherapy for small cell lung cancer. Cancer Treat Rep 69:369–371, 1985.

(*5*) Batist G, Carney DN, Cowan KH, et al: Etoposide (VP-16) and cisplatin in previously treated small-cell lung cancer: Clinical trial and in vitro correlates. J Clin Oncol 4:982–986, 1986.

(*6*) Ochs JJ, Tester WJ, Cohen MH, et al: “Salvage” radiation therapy for intrathoracic small cell carcinoma of the lung progressing on combination chemotherapy. Cancer Treat Rep 67:1123–1126, 1983.

(*7*) Nias AHW: Review. Radiation and platinum drug interaction. Int J Radiat Oncol Biol Phys 48:297–314, 1985.

(*8*) Swartz M, Roseman J, Gunderson L, et al: Podophyllin increases tumor radioresponsiveness. Cancer Chemother Pharmacol 7:238, 1982.

(*9*) Cavalli F: VP-16-213 (etoposide): A critical review of its activity. Cancer Chemother Pharmacol 7:81–85, 1982.

(*10*) Cavalli F, Sonntag RW, Jungi F, et al: VP-16-213 monotherapy for remission induction of small cell lung cancer: A randomized trial using three dosage schedules. Cancer Treat Rep 62:473–475, 1978.

(*11*) Rowland K, Bonomi P, Taylor S, et al: Continuous infusion VP-16, bolus cisplatin, and simultaneous radiation therapy as salvage therapy in small cell bronchogenic carcinoma. *In* Clinical Application of Continuous Infusion Chemotherapy and Concomitant Radiation Therapy (Rosenthal CJ, Rotman M, eds). New York: Plenum Press, 1986, pp 211–216.

Concomitant Therapy With Infusion of Cisplatin and 5-Fluorouracil Plus Radiation in Stage III Non-Small Cell Lung Cancer

Samuel G. Taylor IV,* Anantha K. Murthy, Philip Bonomi, Salitha Reddy, Myung-Sook Lee, L. Penfield Faber, and Douglas J. Mathisen[1]

ABSTRACT—**Based on our experience with head and neck cancer, we have developed an every-other-week, split-course schedule for giving combined cisplatin and 5-fluorouracil infusion and radiation to patients with regionally advanced non-small cell lung cancer for a limited number of cycles prior to planned resection. Sixty-four patients having stage III disease without distant metastases were treated with 4 cycles of combined chemotherapy and radiation to 40 Gy and were offered surgical resection. Thirty-nine patients (61%) underwent surgery. Nine had no residual cancer. No correlation was noted between clinical and histologic responses in the surgery group, but histologic response correlated with subsequent outcome. Survival was 58% at 1 year, 33% at 2 years, and 22% at 3 years. Although encouraging, the overall dismal prognosis of this disease has led us to pursue further improvements in protocol design prior to phase III testing of this concept. To this end, etoposide has been added to the above regimen, extending the cycles from every other week to every third week.**—NCI Monogr 6:327–329, 1988.

Fully 50% of the patients presenting with regionally localized non-small cell lung cancer are considered inoperable due to local tumor extent (T3), presence of pleural effusion, or mediastinal nodal disease (stage III_{M0} disease). These patients have generally had an overall survival of less than 1 year with conventional radiation therapy, with both local control and distant metastases remaining problematic. We have addressed this issue by combining chemotherapy with radiation therapy for a limited number of cycles, followed by surgical resection. Preliminary results including methodology, response to treatment, and toxicity have been reported (*1*).

PATIENTS AND METHODS

Patients with biopsy-confirmed non-small cell lung cancer were considered for study provided that no evidence of metastatic disease existed but tumor extent precluded surgery. Patient selection criteria have been reported (*1*). Briefly, the regional extent qualifying for this study included: 1) gross mediastinal extension of the primary tumor; 2) mediastinal nodal involvement confirmed by thoracotomy, mediastinoscopy, computerized tomography scan; 3) proximal bronchial extension; or 4) chest wall extension. All patients underwent brain tomography scans with infusion, computed tomography of the chest and upper abdomen, and bone nuclide scans.

The simultaneous chemotherapy and radiation treatment included 60 mg cisplatin/m^2 on day 1 (Monday), followed by 800 mg 5-fluorouracil/m^2 over 24 hours on days 1–5 (stops Saturday), and radiation with 2 Gy/day on days 1–5. After a 9-day delay, all treatment was repeated as the next cycle for a total of 4 cycles preoperatively (40 Gy). The radiation fields included the primary tumor and regional nodes. Bilateral supraclavicular nodes, all mediastinal, subcarinal, and ipsilateral hilar nodes were always treated. Contralateral hilar nodes were included when clinically involved. Patients proceeded to surgery provided that no evidence for metastases had developed, the medical condition was acceptable, and the patient agreed. Surgery was planned to include the known disease extent preoperatively.

Survival and time to failure are both taken from the start of therapy until the event. All patients are included in the survival analysis. Three patients who died during the preoperative period and 8 who progressed prior to the time of possible surgery (7 distant and 1 regional) are omitted from some of the analyses of the pattern of failure so that possible bias in those analyses might be avoided. One patient who died clinically disease free but with microscopic disease found at autopsy is included as a relapse at the time of death. We defined complete response as normalization on plain chest x-ray and partial response as greater than 50% reduction in the sum of products of the longest diameter and its perpendicular of all lesions. Stable disease included lesser degrees of response to less than 25% increase in disease. Progression included the development of any new lesions or more than 25% increase in the size of existing lesions.

RESULTS

Sixty-four patients accepted entry into this pilot study and 39 (61%) underwent surgery. Table 1 shows the distribution of various patient characteristics and a comparison of the surgical and nonsurgical groups. Not surprisingly, the nonsurgical group tended to have more patients with worse performance status and more weight loss. There were 14 patients who had T3N0–1 disease and 50 with N2 disease (24 of these T3N2).

Although the combined chemotherapy and radiation regimen usually required hospitalization, patient acceptance was not a problem. Use of a venous access device allowed outpatient treatment in selected patients. Patients actually received between 1 and 6 cycles of treatment. Treatment

---

[1] Department of Medicine, Section of Medical Oncology (*S. G. Taylor IV* and *P. Bonomi*), Department of Radiation Therapy (*A. Murthy, S. Reddy,* and *M-S. Lee*), and Department of Surgery (*L. P. Faber* and *D. J. Mathisen*), Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL.

* *Reprint requests:* Samuel G. Taylor IV, M.D., Section of Medical Oncology, Department of Medicine, Rush-Presbyterian-St. Luke's Medical Center, Suite 830, 1725 W. Harrison St., Chicago, IL 60612.

TABLE 1.—Characteristics of patients with stage III non-small cell lung cancer

Patient characteristics	Percentage of each group		Total
	Surgical $n=39$	Nonsurgical $n=25$	$n=64$
Female sex	36	24	31
Performance status[a]			
0	21	0	13
1	69	68	69
2–3	10	32	19
Predominant histology			
Squamous	33	56	42
Adenocarcinoma	52	16	38
Large cell	15	28	20
T stage			
1	0	8	3
2	38	36	38
3	62	56	59
N stage			
0	10	16	13
1	10	8	9
2	79	76	78
Weight loss, %			
<5	56	44	52
5–10	28	8	20
>10	8	32	17

[a] Performance status criteria of the Eastern Cooperative Oncology Group were used.

was stopped early in 6 patients due to early death of 1 of a myocardial infarction, toxicity in 2, and development of distant metastases in 3. Eleven patients who did not undergo surgery and 8 who had positive mediastinal nodes or positive margins at surgery had 1 or 2 additional cycles of combined chemotherapy and radiation. The actual dose given on average was 91% of the ideal calculated dose for 5-fluorouracil and 76% of that for cisplatin.

With follow-up of 21 to 46 months (median 33 mo), 43 patients (67%) have had recurrences, and 7 (11%) died of other causes [2 of myocardial infarctions, 1 due to toxicity during chemotherapy and radiotherapy, 2 postoperatively (5% operative mortality), 1 of abdominal aneurysm at 13 mo, and 1 of myocardial infarction at 12 mo]. Fourteen patients (22%) remain alive and free of disease.

Of the patients who underwent surgery, 13 (33%) have never had recurrences; this includes 1 patient whose death at 13 months was unrelated to cancer. Eliminating 11 patients in the nonsurgical group who had early recurrence or death, 3 of 14 remaining patients (21%) who either refused surgery or had a medical contraindication to surgery have never had a recurrence, including 1 who died of an unrelated cause at 12 months.

TABLE 2.—Pattern of failure of patients with different histologic types of non-small cell lung cancer

Histologic type	No. of patients	Failures, %	Percentage of all failures: Lung only	Brain only	Other distant	Distant and regional
Squamous	25	64	69	13	19	0
Adenocarcinoma	22	73	25	13	44	19
Large cell	12	92	45	45	9	0

Table 2 lists the sites for those patients whose cancer recurred. Both the frequency of recurrence and the pattern of failure appeared to be influenced by the predominant cell type of non-small cell lung cancer. Whereas squamous cancers tended to recur regionally, adenocarcinomas failed in distant sites 75% of the time, albeit infrequently in brain only, as an isolated site of metastases. Large cell carcinomas were intermediate in lung recurrence but had a disproportionally high (45%) incidence of isolated brain metastases. The only patient with a long-term survival following failure was a patient with large cell carcinoma treated for an isolated brain metastasis with resection and whole-brain radiation therapy. She is alive and well 40 months after the start of her lung treatment and 35 months after the brain recurrence. Twelve surgical patients (31%) had relapses in the lung compared with 9 (64%) of the 14 nonsurgical patients ($P<.05$).

We also examined failure patterns based on initial response to chemotherapy and radiation treatment and disease stage (table 3). Whereas the clinical response to treatment appeared to have no correlation with outcome, complete histologic clearance was associated with improved disease-free survival ($P<.01$). Patients with lower stage N disease (all having T3 disease) tended to have recurrences more frequently (difference not significant). At surgery, histologically positive nodes were found in 3 of 4 patients initially staged as T3N0–1, whereas 12 of 26 patients with stage N2 disease had histologically positive nodes at surgery. There was no difference in rate of relapse based on age, sex, or dose of 5-fluorouracil or cisplatin.

Median survival for all patients was 15 months. For the surgical patients, median survival was 20.8 months compared with 16.5 months for the nonsurgical group, excluding the 11 patients who progressed or died prior to possible surgery.

TABLE 3.—Incidence of relapse based on prior clinical and histologically documented response to chemotherapy and radiation and disease stage[a]

Response and disease stage	No. of patients	Relapse free: No.	%
Clinical			
Complete	5	1	20
Partial	31	10	32
Stable	20	5	25
Pathologic			
Complete	9	6	67
Microscopic only	8	2	25
Gross residual	22	5	23
Initial stage			
N0–1	12	2	17
N2	47	14	30
T1–2	23	8	35
T3	36	8	22

[a] Five patients dying of other causes in the first 4 mo are omitted from this analysis.

DISCUSSION

Radiation therapy alone has resulted in transient palliation but poor control of inoperable non-small cell lung cancer. The Radiation Therapy Oncology Group found a 10% and 14% 2-year, disease-free survival with 40 Gy given as a split course or continuously, respectively (*2*).

Byfield et al. (*3*) used 5-fluorouracil with split-course radiation to give the chemotherapy and radiation simultaneously. Their schedule was different from ours, in that only 2 cycles of chemotherapy and radiation were given with a 3-week delay between cycles. Further radiation to 50 Gy was given continuously without further 5-fluorouracil. They reported dose-limiting toxicity from severe mucositis and esophagitis at 5-fluorouracil doses of 30 to 35 mg/kg (problems not encountered in our experience when using a lower dose). Their reported survival for stage III_{M0} patients was 9.6 months.

The treatment regimen used in this trial was based on encouraging results in head and neck cancer (*4*). Our results with cisplatin, 5-fluorouracil, and radiation, given concomitantly, compare favorably with the above studies. Patients who underwent surgery had a median survival of 20.8 months. We emphasize, however, that this is a select population. The overall survival for all patients was 15 months. If patients who died or progressed prior to being able to undergo surgery were excluded, the median survival of the remaining nonsurgical group was 16.5 months. Bias from imbalance of other prognostic factors and small numbers could account for any differences.

Neither clinical response nor initial T and N staging correlated with histologic response or therapeutic outcome. In contrast, histologic response did appear important in the prediction of subsequent recurrence. The clearest value of surgery at this time is in providing tissue for histologic evaluation.

The pattern of failure indicated that failure in the lung or mediastinum remains a major problem especially for squamous cell carcinomas. Failure in the brain was common for large cell carcinomas and adenocarcinomas as observed by others (*5*). However, only with large cell carcinomas was this commonly the only site of failure. Surgery did correlate with fewer lung and mediastinal relapses (31% vs. 64%).

We conclude that concomitant radiation and chemotherapy offers an appealing approach. We have achieved disease control and median survival rates that have been encouraging, with acceptable toxicity and patient tolerance, compared with other chemotherapy and radiation therapy schedules. However, cure rates remain dismal, with 22% of the patients remaining disease free at 3 years. Consequently, we believe further exploration of this approach of adding other effective antineoplastic drugs with possible radiation potentiation is indicated. To this end, we have added etoposide, extending the rest period between cycles to 2 weeks, as reported elsewhere (*6*). Surgical resection with microscopic evaluation of response is necessary for clinicians to establish individual benefit from treatment. Histologic complete response is associated with improved disease-free survival. Directors of other treatment programs should aim to improve on the percentage of such complete responses.

REFERENCES

(*1*) Taylor SG IV, Trybula M, Bonomi PD, et al: Simultaneous cisplatin/5-FU infusion and radiation followed by surgical resection in regionally localized stage III, non-small cell lung cancer. Ann Thorac Surg 43:87–91, 1987.

(*2*) Perez CA, Stanley K, Rubin P, et al: Patterns of tumor recurrence after definitive irradiation for inoperable non-oat cell carcinoma of the lung. Int J Radiat Oncol Biol Phys 6:987–994, 1980.

(*3*) Byfield JE, Stanton W, Sharp TR, et al: Phase I–II study of 120-hour infused 5-FU and split-course radiation therapy in localized non-small cell lung cancer. Cancer Treat Rep 67:933–936, 1983.

(*4*) Taylor SG IV, Murthy AK, Showel JL, et al: Improved control in advanced head and neck cancer with simultaneous radiation and cisplatin/5-FU chemotherapy. Cancer Treat Rep 69:933–939, 1985.

(*5*) Choi NCH, Grillo HC, Gardiello M, et al: Basis for new strategies in postoperative radiotherapy of bronchogenic carcinoma. Int J Radiat Oncol Biol Phys 6:31–35, 1980.

(*6*) Bonomi P, Rowland KM Jr, Taylor SG IV, et al: Phase II trial of therapy with etoposide, 5-fluorouracil by continuous infusion, cisplatin, and simultaneous split-course radiation in stage III non-small cell bronchogenic carcinoma. NCI Monogr 6:331–334, 1988.

Phase II Trial of Therapy With Etoposide, 5-Fluorouracil by Continuous Infusion, Cisplatin, and Simultaneous Split-course Radiation in Stage III Non-Small Cell Bronchogenic Carcinoma

Philip Bonomi,[1,*] Kendrith M. Rowland, Jr.,[2] Samuel G. Taylor IV,[1] Mitchell Pincus,[1] Salitha Reddy,[1] Myung-Sook Lee,[1] L. Penfield Faber,[1] William Warren,[1] and C. F. Kittle[1]

ABSTRACT—**Survival of patients who have clinical stage $\mathrm{III}_{\mathrm{M0}}$ non-small cell bronchogenic carcinoma remains relatively short despite treatment with either surgery or radiation. Results from a phase II study of simultaneous continuous infusion of 5-fluorouracil, cisplatin, and split-course radiation with or without surgery indicate that median survival duration in patients treated with this combined modality approach may be better than the median survival for patients treated with radiation alone. Etoposide has been added to this regimen, and 32 stage $\mathrm{III}_{\mathrm{M0}}$ non-small cell lung cancer patients have been treated with the 3-drug regimen resulting in a 73% clinical partial remission rate. No residual tumor was found in 6 of 12 patients who had pulmonary resection after 4 courses of chemotherapy and radiation. The sites of failure in 8 patients with recurrent disease are as follows: local only, 3; distant only, 4; and local and distant, 1. The major toxicities have been leukopenia, nausea, and vomiting. The median leukocyte nadir was 2,400/mm^3. A leukocyte count less than 1,000/mm^3 was observed in 2 patients (7%), 1 of whom died of progressive pneumonia. All patients experienced nausea, vomiting, and anorexia. Severe esophagitis, dermatitis, and pneumonitis were not observed. Survival analysis has not been done because median follow-up time (326 days) is relatively short.—NCI Monogr 6:331-334, 1988.**

Approximately one-third of the patients who have non-small cell lung cancer will present with locally advanced disease and no apparent distant metastases. Although thoracic radiation provides palliation for many of them, few will be cured by radiation (*1,2*) or by pulmonary resection alone (*3*).

Recent reports show that response rates for combination chemotherapy regimens in patients with locally advanced disease appear to be approximately double (*4–6*) the response rate observed in patients with distant metastases (*7*). However, the median survival durations observed in nonrandomized trials of sequential chemotherapy–radiation studies (*5,6*) show no apparent advantage over the 9- to 12-month median survival observed with thoracic radiation alone (*1,2*).

The disappointing survival results observed with the use of sequential chemotherapy–radiation led to our trying a modification of the simultaneous use of continuous infusion 5-fluorouracil and split-course radiation described by Byfield et al. (*8*). Cisplatin was added to 5-fluorouracil and radiation because of 1) its radiation enhancing effect (*9*) and 2) the relatively high response rate and acceptable toxicity observed in head and neck cancer patients treated with the 2-drug regimen and split-course radiation described by Taylor et al. (*10*).

Use of this combination treatment for 64 clinical stage $\mathrm{III}_{\mathrm{M0}}$ non-small cell lung cancer patients resulted in a 15-month median survival (*11*), which compares favorably with the 9- to 12-month median survival reported for radiation alone (*2,3*). Similarly, 35% of the patients treated with the combined modality regimen survived for 2 years compared with 15%–20% of patients treated with radiation alone (*2,3*).

Encouraged by these initial observations, we added etoposide for the following reasons: 1) Etoposide and cisplatin had produced a 56% response rate in patients with limited non-small cell lung cancer (*4*), and 2) a trial in small cell lung cancer had demonstrated acceptable toxicity with simultaneous etoposide and cisplatin and radiation (*12*). Experience with the simultaneous use of etoposide, cisplatin, and split-course radiation in small cell lung cancer patients (*13*) showed that the addition of etoposide would require a longer interval between treatments to allow recovery from myelosuppression.

The preliminary results observed with 5-fluorouracil–cisplatin–etoposide–radiation trial are described in this report.

PATIENTS AND METHODS

Patients who had non-small cell lung cancer confirmed either histologically or by cytology obtained from bronchial brushing or from lung or lymph node needle biopsy were eligible for this study. Sputum cytology specimens were not considered adequate.

Either histologic or clinical evidence of stage $\mathrm{III}_{\mathrm{M0}}$ disease was required for inclusion in this trial. Criteria for disease classification as clinical stage $\mathrm{III}_{\mathrm{M0}}$ included tumor invasion of the mediastinum or chest wall depicted on CAT scan and/or the presence of mediastinal nodes greater than

---

ABBREVIATIONS: CAT = computerized axial tomography; ECOG = Eastern Cooperative Oncology Group.

---

[1] Department of Medicine, Section of Medical Oncology (*P. Bonomi* and *S. G. Taylor IV*), Department of Radiation Therapy (*M. Pincus, S. Reddy,* and *M-S. Lee*), and Department of Cardiovascular-Thoracic Surgery (*L. P. Faber, W. Warren,* and *C. F. Kittle*), Rush Medical College, Chicago, IL.

[2] Section of Medical Oncology, Carle Clinic Association, Urbana, IL.

[*] *Reprint requests:* Philip Bonomi, M.D., Section of Medical Oncology, Department of Medicine, Rush Medical College, 1743 W. Harrison St., Chicago, IL 60612.

2 cm in diameter on the scan. Evidence of distant metastases excluded a patient from this study. Prior to starting treatment, each patient had a nuclide bone scan, CAT brain scan with Hypaque infusion, and a CAT scan of the chest and upper abdomen. All patients gave informed written consent.

Other eligibility requirements included: performance status (ECOG) of 3 or less, leukocyte count over 4,000/mm^3, platelet count over 100,000/mm^3, BUN less than 25 mg/dl, serum creatinine less than 1.5 mg/dl, and bilirubin under 1.5 mg/dl. Physical examination, CBC, platelet count, SMA18, and chest x-ray were done before each course of treatment.

Each patient was initially evaluated by a thoracic surgeon who classified the patient as potentially resectable or nonresectable regardless of response to treatment. Patients who were considered potentially resectable were reevaluated 4 weeks after the fourth course of treatment.

The chemotherapy regimen was as follows: 60 mg cisplatin/m^2 iv over 1 hour on day 1, 60 mg etoposide/m^2 iv over 1 hour on days 1–4, and 800 mg 5-fluorouracil/m^2 iv over 24 hours for 4 consecutive days. For patients whose ECOG performance status was 2–3 or who lost more than 10% of their usual body weight, the starting doses of etoposide and 5-fluorouracil were 45 and 600 mg/m^2, respectively. Treatment was repeated every 21 days if toxicity permitted.

Only the doses of these 2 drugs (not the cisplatin) were modified for hematologic toxicity. The cisplatin dose was modified for renal toxicity. All 3 drugs were reduced for moderate or severe nausea and vomiting.

Radiation therapy was given on days 1–5 of each 21-day cycle. Patients who were classified as potentially resectable received 200-cGy fractions in a single daily treatment on days 1–5. Treatment was stopped when a total dose of 40 Gy was given. Initially, 10 patients who were classified as nonresectable received 2 fractions of 116 cGy separated by at least 4 hours on days 1–5 of each cycle. More recently, all patients have received 200 cGy as a single daily treatment.

Only supervoltage equipment was used. The fields included the primary tumor with a 1- to 2-cm minimum margin and regional lymph nodes. Bilateral supraclavicular nodes and all mediastinal, subcarinal, and ipsilateral hilar nodes were always treated. The contralateral hilar nodes were included when clinically involved. All doses were calculated at the midplane of the target volume, and minimal treatment distances to the isocenter were at least 80 cm. We used the anterior/posterior–posterior/anterior parallel-opposed technique for the first 40 Gy, followed by obliqued fields to avoid totally the spinal cord, thereby treating only the initial gross disease with a 1-cm margin. In the hyperfractionated group, a spinal cord block was added after 40 Gy and obliqued field reduction after 44 Gy. Tissue compensators were not used. Doses to the lower cervical and upper thoracic spinal cord were calculated on several patients and found to be in the range of 110%–115% of the isocenter dose, yielding an effective maximum spinal cord dose of approximately 4,500 cGy.

Responses were defined according to ECOG criteria (*14*).

RESULTS

Thirty of the 32 patients entered on this protocol could be evaluated for response and toxicity. Of the 2 patients excluded from response and toxicity analyses, 1 had a history of ventricular arrhythmias and died suddenly before the second course of treatment could be started; the other patient was removed from the study after suffering a myocardial infarction on the first day of treatment. Patient characteristics are summarized in table 1. Results of pretreatment staging are as follows: T3N2, 20 patients (62%); T3N0, 9 patients (29%); and T1-2N2, 3 patients (9%).

Twenty-two of 30 patients (73%) have been classified as having a partial remission. Thoracotomy has been performed in 12 patients resulting in 6 pneumonectomies, 4 lobectomies, and 2 segmental resections combined with resection of the adjacent chest wall. Histologic examination of the resected specimen has shown the following results: no residual neoplasm in the resected lung or lymph nodes, 6 patients (50%); residual microscopic foci of tumor, 3 patients (25%); and gross residual disease with tumor-free margins, 3 patients (25%). Eighteen patients (60%) did not proceed to thoracotomy for the following reasons: inadequate cardiopulmonary function, 4 (13%); patient refusal, 2 (6%); tumor progression, 1 (3%); surgery judged as technically impossible by thoracic surgeon, 11 (33%).

Progressive disease has been observed in 8 patients, with a median follow-up time of 326 days. The sites of failure include: infield recurrence only, 3 patients; distant disease only, 4 patients; and infield and distant, 1 patient. Brain metastases have been detected in 2 patients. One instance of infield recurrence only and two instances of distant disease only were observed during treatment with the 3-drug plus radiation regimen.

Radiation given as 2 daily 116-cGy fractions was initiated in 10 patients who were classified as nonsurgical candidates. Five patients did not complete the planned course of treatment for the following reasons: appearance of distant metastases, 2 patients; myocardial infarction or cardiac arrhythmia, 2 patients; or death due to aspiration pneumonia, 1 patient. An additional group of 8 patients who were classified as ineligible for surgery received 200 cGy as a single daily fraction. The dose of radiation in the patients who completed hyperfractionated treatment was 59 to 67 Gy with a median of 61 Gy, and in the patients who received single daily fractions, the dose was 26 to 60 Gy with a median of 58 Gy. The patient who received 26 Gy had been treated with preoperative radiation 6 years earlier for another primary lung cancer. All 5 patients who received hyperfractionated treatment had partial remis-

TABLE 1.—Patient characteristics[a]

Parameter	No. of patients	%
Males	21	66
Females	11	34
ECOG performance status		
0–1	24	75
2–3	8	25
Previous weight loss		
>0<5%	16	50
>5<10%	6	18
>10%	8	25
Unknown	2	7
Histology		
Squamous cell	16	50
Adenocarcinoma/large cell	16	50

[a] The median age of patients was 55 yr, with a range of 37–74 yr.

sions compared with 7 of 8 patients who received single daily fractions. There was one infield recurrence in each group.

Ten of 32 patients died, 4 of progressive tumor, 1 of treatment-related sepsis, 3 of acute cardiovascular events, 1 of pneumonia which occurred 3 months after completion of treatment, and 1 of aspiration pneumonia associated with a tumor partially obstructing her esophagus. Survival analysis is not included in this report because of the relatively short follow-up.

Median leukocyte count and platelet count nadirs were 2,400/mm^3 and 241,000/mm^3, respectively. Leukocyte counts less than 1,000/mm^3 and platelet counts less than 30,000/mm^3 were observed in the same 2 patients.

Both of the patients whose leukocyte count was less than 1,000/mm^3 developed infections, and 1 died despite antibiotic therapy; this death is classified as being treatment related. There were no other episodes of treatment-related leukopenia and infection or any episodes of treatment-related thrombocytopenia and bleeding.

All patients experienced some degree of anorexia, nausea, or vomiting. Five patients (16%) lost from 10 to 15 pounds during treatment. The median value for the difference between initial weight and weight observed 4 weeks after completion of chemotherapy–radiation was a gain of 8 pounds.

There were no instances of stomatitis or of moderate to severe esophagitis. All patients had significant alopecia, and all experienced fatigue.

Serum creatinine rose to 1.7 mg/dl in 2 patients, but no other patient experienced a serum creatinine greater than 1.5 mg/dl.

Cough, mild dyspnea, and radiographic findings consistent with radiation pneumonitis were observed in 4 patients; 2 were treated with indomethacin and 2 received prednisone. All have improved, and the steroid or nonsteroidal agent has been discontinued. One of these patients subsequently developed a lung abscess that has responded to antibiotics. All patients had erythema of the skin in the radiation portal, but none had moist desquamation.

At the time of surgical resection, dense fibrous tissue was encountered, and this frequently made the dissection of hilar structures difficult. Three of these surgical patients have developed empyema from which each has recovered. One of them required an emergency thoracotomy for postsurgical hemorrhage that was not related to thrombocytopenia. There have been no postoperative deaths.

DISCUSSION

Although the 73% clinical response rate observed with 5-fluorouracil–cisplatin–etoposide and radiation therapy is similar to the response rate reported for sequential chemotherapy–radiation trials (*5,6*), perhaps the frequency of histologic complete response rate is a better indicator of the efficacy of this combined modality treatment. In the trial without etoposide (*11*), surgical patients whose resected specimens contained no tumors had a lower recurrence rate than did patients whose resected specimens contained tumors, i.e., 33% versus 59%, respectively. The 6 (50%) histologic complete responses in 12 patients receiving the 3 drugs plus irradiation compare favorably with the 23% histologic complete response rate observed in 39 who were not given etoposide (*11*).

At this point, it is impossible for us to determine whether there is a definite trend for a higher histologic complete remission rate with the 3-drug regimen and irradiation. Equally difficult is a comparison of histologic complete remission rates for this combined modality trial and for trials of preoperative radiation alone, one of which resulted in a 46% histologic complete remission rate (*15*). However, this study (*15*) included patients whose disease was staged as less than stage $\mathrm{III}_{\mathrm{M0}}$.

One of the two major toxicities observed with this treatment was leukopenia. Although leukopenia may have been underestimated because midcycle counts were not done routinely, the median leukocyte count of 2,400/mm^3 at the start of each treatment course shows that, in general, leukopenia was not severe. Two patients experienced febrile episodes while their leukocyte counts were less than 1,000/mm^3, and 1 patient died despite treatment with antibiotics and increased leukocytes to 6,100/mm^3 within 5 days. The ECOG performance status in these 2 patients was 2–3, but 1 had lost more than 10% of his usual body weight. These observations have resulted in our reducing the doses of etoposide and 5-fluorouracil to 75% of the usual starting doses in patients whose ECOG performance status is 2–3 or whose weight is 10% lower than usual.

Comparison of myelosuppression observed with the 2 regimens (*11*) shows that 5% had WBC counts less than 2,000/mm^3 on 2 drugs versus 16% on 3 drugs and 29% had WBC counts less than 3,000/mm^3 on 2 drugs versus 62% on 3 drugs.

Anorexia, nausea, and vomiting were major toxicities during treatment with 5-fluorouracil–cisplatin–etoposide–radiation therapy, and 5 (16%) patients lost from 10 to 15 pounds during treatment. Despite gastrointestinal side effects during each 5-day course of treatment, by the end of 4–6 courses the median weight change was an increase of 8 pounds.

Four (12%) of our patients developed mild radiation pneumonitis following treatment. Although 3 of 12 patients developed infection after pulmonary resection, there has been no operative mortality.

In conclusion, the 3-drug plus radiation therapy produces a relatively high clinical response rate in patients with locally advanced non-small cell lung cancer. Similarly, preliminary observations have shown a 50% rate of histologically confirmed complete response in surgical specimens. Toxicity from combining the 3 drugs and simultaneous split-course thoracic radiation is moderate with leukopenia, anorexia, nausea, and vomiting being the major toxic effects. Esophagitis and radiation pneumonitis have not been significant problems. Although pulmonary resection is difficult because of dense fibrous tissue, surgery following 4 courses (4,000 cGy) and a 4-week rest period does not appear to be associated with an increased rate of operative mortality. More follow-up is needed if we are to determine the median survival duration and the 2-year survival rate for patients receiving this treatment.

REFERENCES

(*1*) Perez CA, Stanley K, Rubin P, et al: A prospective randomized study of various irradiation doses and fractionated schedules in the treatment of inoperable non-oat cell carcinoma of the lung. Cancer 45:2744–2753, 1980.

(*2*) Petrovich Z, Stanley K, Cox JD, et al: Radiotherapy in the management of locally advanced lung cancer of all cell types. Cancer 48:1335–1340, 1981.

(*3*) Mountain CF: Assessment of the role of surgery. Ann Thorac Surg 24:365–373, 1977.

(*4*) Longeval E, Klastersky J: Combination chemotherapy with cisplatin and etoposide in bronchogenic squamous cell carcinoma and adenocarcinoma. Cancer 50:2751–2756, 1982.

(*5*) Fram R, Skarin A, Balikian J, et al: Combination chemotherapy followed by radiation therapy in patients with regional stage III unresectable non-small cell lung cancer. Cancer Treat Rep 69:587–590, 1985.

(*6*) Wagner H, Ruckdeschel J, Bonomi P, et al: Treatment of locally advanced non-small cell lung cancer (NSCLC) with mitomycin C, vinblastine, and cisplatin (MVP) followed by radiation therapy: An ECOG pilot study. Proc ASCO 4:183, 1985.

(*7*) Ruckdeschel JC, Finkelstein DM, Mason BA, et al: Chemotherapy for metastatic non-small cell bronchogenic carcinoma: EST 2575, generation V—A randomized comparison of four cisplatin containing regimens. J Clin Oncol 3:72–79, 1985.

(*8*) Byfield JE, Sharp RT, Frankel SS, et al: Phase I and II trial of five-day infused 5-fluorouracil and radiation in advanced cancer of the head and neck. J Clin Oncol 2:406–413, 1984.

(*9*) Douple EB, Richmond RC: Platinum complexes as radiosensitizers of hypoxic mammalian cells. Br J Cancer 37(suppl III):98–102, 1978.

(*10*) Taylor SG IV, Murthy AK, Showel JL, et al: Improved control in advanced head and neck cancer with simultaneous radiation and cisplatin/5-FU chemotherapy. Cancer Treat Rep 69:933–939, 1985.

(*11*) Taylor SG IV, Trybula M, Bonomi PD, et al: Simultaneous cisplatin/5-FU infusion and radiation followed by surgical resection in regionally localized stage III non-small cell lung cancer. Ann Thorac Surg 43:87–91, 1987.

(*12*) Murray N, Jadzic E, Shah B, et al: Alternating chemotherapy and thoracic radiotherapy with concurrent cisplatin for simultaneous small cell carcinoma of the lung. Proc ASCO 3:214, 1984.

(*13*) Rowland K, Bonomi P, Taylor SG IV, et al: Continuous infusion VP16, bolus cisplatin, and simultaneous radiation therapy as salvage therapy in small cell bronchogenic carcinoma (SCBC). Proc ASCO 5:181, 1986.

(*14*) Oken MM, Creech RH, Tormey DC, et al: Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649–655, 1982.

(*15*) Bromley LL, Szur L: Combined radiotherapy and resection for carcinoma of the bronchus. Lancet 1:937–941, 1955.

Combination of Chemotherapy and Radiotherapy in Limited Small Cell Lung Carcinoma: Results of Alternating Schedule in 109 Patients

T. Le Chevalier,[1,*] R. Arriagada,[1] H. de Thé,[1] H. De Cremoux,[2] M. Martin,[2] P. Baldeyrou,[1] P. Ruffié,[1] F. Benna,[1] M. L. Cerrina,[3] H. Sancho-Garnier,[1] and M. Hayat[1]

ABSTRACT—One hundred nine patients with limited small cell lung carcinoma were entered in a phase II study of treatment consisting of alternating 6 cycles of combination chemotherapy and 3 courses of mediastinal radiotherapy. Chemotherapy consisted of 40 mg doxorubicin/m^2 on day 1; 75 mg etoposide/m^2 on days 1, 2, and 3; 300 mg cyclophosphamide/m^2 on days 3, 4, 5, and 6; and 400 mg methotrexate/m^2 on day 2 (plus folinic acid rescue) or 100 mg cisplatin/m^2 on day 2. The total mediastinal radiation dose was 45 or 55 Gy. A 6- to 8-cycle maintenance chemotherapy followed this induction protocol. The complete remission rate at the end of the induction therapy was 79%. The local recurrence rate was 25%, and the distant metastases rate was 52%. Median survival is 17.2 ± 1.2 months and survival rate at 3 years is 26%. Lethal toxicity occurred in 3% of the patients, and long-term survivors are being evaluated. Our results justify further investigations with this alternating schedule.—NCI Monogr 6:335–338, 1988.

Whereas CT is largely accepted as the treatment of choice in SCLC, the role of thoracic RT has been much debated (*1–7*). No consensus has been reached, although RT is the single most effective agent in SCLC. To avoid the toxicity of concomitant therapy, Tubiana et al. (*8*) proposed alternating multiple short courses of RT and CT.

The use of such a schedule in the treatment of limited SCLC was started at the Institut Gustave-Roussy in 1980. The rationale of this approach has been discussed elsewhere (*9*). In summary, we believed that RT should be incorporated into the induction treatment of this disease. The principal cause of failure in SCLC is the appearance of resistant cells to the treatment. Goldie and Coldman (*10*) developed a mathematical model that supports the use of alternating regimens of non-cross-resistant CT. However, this approach has not been proved valuable in randomized clinical trials in SCLC. The likely reason for this is that the drugs used had some degree of cross-resistance. The mechanisms of drug cross-resistance are currently not well understood, and this fact has limited the use of this approach. Both RT and CT are agents without cross-resistance and, if their administration is well planned, there will be minimal cross toxicity (*8,11*). In the planning of alternating RT–CT schedules, we thought that: 1) The CT should be initiated immediately after the histologic diagnosis has been made and staging investigations completed; 2) CT should be administered regularly every month during the whole induction treatment; 3) thoracic RT should be given early in the schedule to the maximal dose tolerated and should not interrupt the CT, thereby reducing the risk of resistant cells developing; and 4) a 7-day interval between CT and RT was justified to avoid the increased toxicity associated with concurrent therapy (*12–15*).

Our preliminary results (*16*) were satisfactory in patient tolerance and relapse-free survival (32% at 2 yr), and we introduced two modifications. Methotrexate was replaced by cisplatin to decrease hematologic toxicity, and the total dose of thoracic RT was increased from 45 to 55 Gy. It seemed important to us to reach the maximum tolerance dose, unknown for this kind of combination, in an attempt to optimize the results. We report here the results obtained in 109 consecutive patients with limited SCLC treated with these 2 alternating protocols.

PATIENTS AND METHODS

One hundred nine patients with limited disease were treated from May 1980 to December 1984. Patients had received no prior RT, no more than 1 course of CT, and had histologically proven SCLC. Staging investigations included chest x-ray, full blood count, chemistries, fiberoptic bronchoscopy, bone marrow biopsy, bone scan, liver ultrasound or computerized axial tomography, and brain scan or computerized axial tomography scan. All patients were less than 70 years old, and their Karnofsky performance status was more than 40%.

The treatment schedule is shown in figure 1. Initially, RT was delivered in anterior and posterior fields in 2 courses of 15 Gy in 6 fractions over 10 days (protocol 002) or 20 Gy in 8 fractions over 14 days (protocol 004); the target volume included all the initially visible tumor (with a 1.5-cm margin of normal lung tissue) as well as the mediastinum and both hilar and supraclavicular regions. A third course of RT was given in two anterior–posterior (002) or lateral (004) fields that spared the spinal cord and delivered 15 Gy in 6 fractions over 10 days. Prophylactic cranial irradiation was given in 10 fractions over 14 days for a total of 30 Gy during the first course of thoracic RT.

Patients in CR were given maintenance CT consisting of 6 to 8 alternating cycles of 2 drug regimens as follows:

ABBREVIATIONS: CT = chemotherapy; SCLC = small cell lung cancer; RT = radiation therapy; CR = complete remission(s); PR = partial remission.

[1] Institut Gustave-Roussy, Villejuif, France.

[2] Hôpital Intercommunal, Creteil, France.

[3] Hôpital Antoine Béclère, Clamart, France.

[*] *Reprint requests:* Thierry Le Chevalier, M.D., Comité de Pathologie Thoracique, Institut Gustave-Roussy, rue Camille Desmoulins, 94800 Villejuif Cedex, France.

Regimen	Day(s)	Drug dosage/m^2
1	1	40 mg doxorubicin
		or
		5 mg mechlorethamine
	1–2	20 mg methotrexate
	2	1 mg vincristine
2	1–2	200 mg etoposide
	2	25–50 mg lomustine
		or
	3–5	300 mg cyclophosphamide
	3–10	100 mg procarbazine

Four patients included in this report, who were in CR after induction therapy and who received intensive CT with autologous bone marrow transplantation as consolidation treatment instead of maintenance CT, have also been reported elsewhere (*17*).

All patients were restaged following the sixth cycle of induction CT and at completion of maintenance CT. Restaging was identical to the initial assessment.

Criteria for tumor response were as follows: CR: complete disappearance of all objective tumor on the chest x-ray, visually and histologically at the time of fiberoptic bronchoscopy and without evidence of new disease; PR: decrease of 50% or more of the product of the two largest perpendicular diameters of radiologically measurable disease with concurrent endoscopic verification and without evidence of new disease; stable disease: decrease of less than 50% of the product of the two largest perpendicular diameters of measurable disease radiologically with concurrent endoscopic verification and without evidence of new disease; progressive disease: radiologic or endoscopic progression or evidence of new disease.

Treatment toxicity was registered according to the World Health Organization grading scale (*18*).

Chest x-rays were regularly taken and any minor change was further investigated by fiberoptic bronchoscopy, in addition to those radiographs planned in the protocol.

Prognostic factors were analyzed by a multiple regression model as described by Cox (*19*).

Our population consisted of 96 men and 13 women and the mean age was 53.9 years. Mean performance status according to the Karnofsky index was 81.7% (range 40%–100%).

Two patients presented with stage I disease, 5 with stage II, and 102 with stage III. Eight patients had a supraclavicular lymph node involvement, and 5 had a paraneoplastic syndrome.

TABLE 1.—Therapeutic results[a]

Patients' status	No. of patients	%
PR > 50% after 2 courses of CT	95	87
CR after induction treatment	86	79
PR after induction treatment	9	8
Local recurrence without distant metastases	13	12
Distant metastases without local recurrence	43	39
Local recurrence plus distant metastases	14	13

[a] Of the 109 patients, 102 (94%) completed induction treatment.

RESULTS

Response rate to chemotherapy was assessed on the chest x-ray done 10 days after the second cycle of chemotherapy. Seven patients did not complete the induction protocol because of toxicity (3) or disease progression (4). Therapeutic results are summarized in table 1. Twenty-seven patients had local recurrence after CR and 57 presented with secondary metastases.

Survival rate is reported on figure 2. Median overall survival is 17.2 ± 1.2 months and median disease-free survival is 14.7 ± 1.1 months. Currently, 19 patients have been followed for more than 3 years. Mean performance status of these long-term survivors is 90%. One patient presented with a second cancer (bladder carcinoma).

Protocols 002 and 004 showed no statistical differences in response rate and survival.

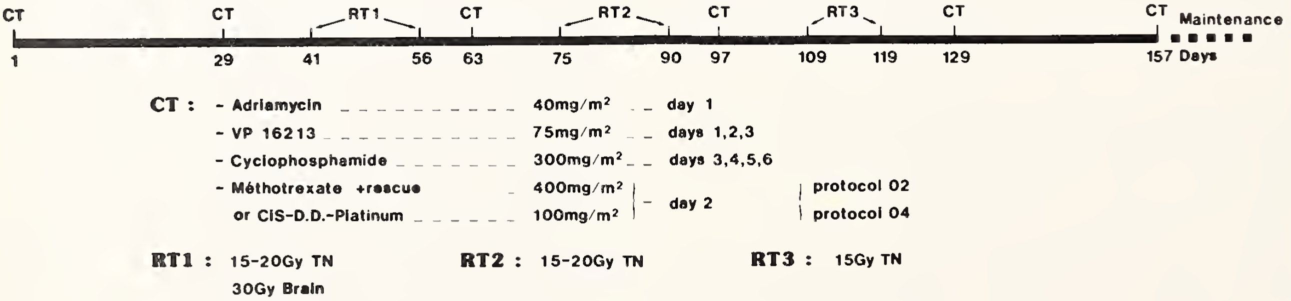
Acute Toxicity

Three patients died of aplasia during the induction therapy (2 in protocol 002 and 1 in protocol 004). Hematologic toxicity was observed in all patients; it was grade IV in 29%, and it was more frequently observed following the fourth cycle of chemotherapy.

All patients who received cisplatin exhibited nausea and vomiting. A reversible renal and neurologic toxicity (grade I) was observed in 1 patient. Moderate esophagitis was seen in 7% of the patients and pneumonitis in 11%.

Delayed Toxicity

One patient died of persistent bone marrow hypoplasia. Pulmonary fibrosis was observed on chest x-ray in most of the patients, but it was symptomatic in only 7%. Pericardial effusion was found in 50% of the patients surviving more than 3 years but it was symptomatic in only 1 patient.



CT : - Adriamycin .... 40mg/m^2 .. day 1
- VP 16213 .... 75mg/m^2 .. days 1,2,3
- Cyclophosphamide .... 300mg/m^2 .. days 3,4,5,6
- Méthotrexate +rescue . 400mg/m^2 – day 2 protocol 02
or CIS-D.D.-Platinum .... 100mg/m^2 – day 2 protocol 04

RT1 : 15-20Gy TN
30Gy Brain

RT2 : 15-20Gy TN

RT3 : 15Gy TN

FIGURE 1.—Treatment schedule. VP16213 = etoposide; TN = tumor, mediastinal and supraclavicular lymph nodes.

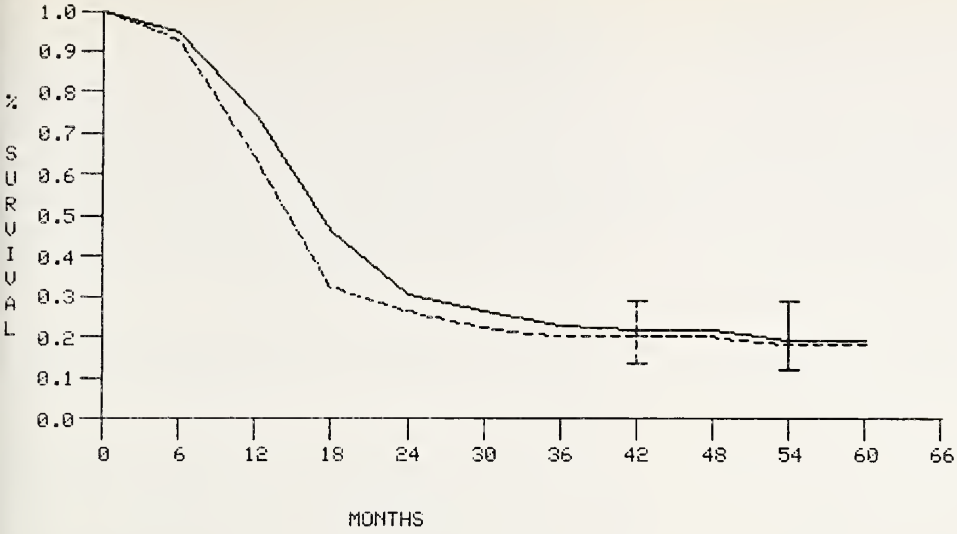


FIGURE 2.—Global (*solid line*) and disease-free (*dashed line*) survival.

Prognostic Factors

Protocols 002 and 004 were analyzed separately because of the differences in induction therapy (methotrexate vs. cisplatin and 45 Gy vs. 55 Gy delivered to the tumor and mediastinum).

We analyzed age, sex, tumor size, performance status, and drug doses/square millimeter actually administered during the first course of chemotherapy, mean dose per course, and initial hematologic toxicity to determine their prognostic incidence in these trials.

The major prognostic factors were hematologic toxicity of the first course of CT ($P<.03$) in protocol 002 (*20*) and the actual delivered doses of cyclophosphamide ($P<.001$) and cisplatin ($P<.002$) during the first course of CT in protocol 004 (*21*).

DISCUSSION

The benefits of combined chemotherapy and radiation therapy are counterbalanced by increased toxicity. In malignant diseases such as SCLC with a high propensity to metastasize distantly, oncologists have hesitated to interrupt CT to deliver thoracic RT. However, CT alone is insufficient to obtain the high rate of prolonged local control (*22-24*) necessary for long-term survival. Often, RT is given in 1 short course of a moderate dose (*1,25*). This approach has certainly improved the percentage of complete responses, but it has not been adequate to ensure long-term local control (*2,13*).

These protocols were designed to deliver relatively high-dose RT in 3 courses alternating with combination CT to maximize both local control and long-term survival in patients with limited disease. The relatively high performance status in our series reflects the (apparently) limited character of the disease. The first protocol was initiated in 1980 and was completed 18 months later. At that time, we observed a relatively severe hematologic toxicity and a local recurrence rate of 17% at 1 year. These observations led us to substitute cisplatin for methotrexate and to increase the total dose of thoracic RT.

This study has proved the feasibility of these 2 protocols provided that the coordination between involved oncologists is high. Acute toxicity and the late complication rate appear to be acceptable.

Our results confirm the survival benefit obtained in limited stage SCLC with RT–CT combinations as reported by other authors (*26,27*). It appears that alternating schedules give a high level of CR with a low rate of local recurrences and avoid the major toxicity of concomitant RT and CT administration (*28*).

The study of prognostic factors points out the major importance of the first CT cycle in induced hematologic toxicity in protocol 002 or of drug doses in protocol 004. These data have led us to design a phase II study in which 1) initial doses of CT are increased up to 75 $\text{mg}\cdot\text{m}^2\cdot\text{day}^{-1}$ over 5 days for etoposide and up to 30 $\text{mg}\cdot\text{m}^2\cdot\text{day}^{-1}$ over 5 days for cisplatin; 2) the first course of RT is delivered after the first course of CT, thereby preventing development of early resistant cells (*11*); and 3) the total dose of RT is increased up to 61 Gy in 3 courses.

In conclusion, alternating CT and RT seems to be a promising approach to the treatment of limited SCLC. Many different combinations of doses could be envisioned with similar schedules.

REFERENCES

(*1*) BLEEHEN NM, BUNN PA, COX JD, et al: Role of radiation therapy in small cell anaplastic carcinoma of the lung. Cancer Treat Rep 67:11–19, 1983.

(*2*) BYHARDT RW, COX JD: Is chest radiotherapy necessary in any or all patients with small cell carcinoma of the lung? Yes. Cancer Treat Rep 67:209–215, 1983.

(*3*) BYHARDT RW, COX JD, HOLOYE PY, et al: The role of consolidation irradiation in combined modality therapy of small cell carcinoma of the lung. Int J Radiat Oncol Biol Phys 8:1271–1276, 1982.

(*4*) COHEN MH: Is thoracic radiation therapy necessary for patients with limited-stage small cell lung cancer? No. Cancer Treat Rep 67:217–221, 1983.

(*5*) COX JD, HOLOYE PY, BYHARDT RW, et al: The role of thoracic and cranial irradiation for small cell carcinoma of the lung. Int J Radiat Oncol Biol Phys 8:191–196, 1982.

(*6*) PEREZ CA, KRAUSS S, BARTOLUCCI AA, et al: Thoracic and elective brain irradiation with concomitant or delayed multiagent chemotherapy in the treatment of localized small cell carcinoma of the lung: A randomized prospective study by the Southeastern Cancer Study Group. Cancer 47:2407–2413, 1981.

(*7*) WHITE JE, CHEN T, MCCRACKEN J, et al: The influence of radiation therapy quality control on survival, response and sites of relapse in oat cell carcinoma of the lung. Preliminary report of a Southwest Oncology Group study. Cancer 50:1084–1090, 1982.

(*8*) TUBIANA M, ARRIAGADA R, COSSET JM: New types of fractionation for optimization of combinations of radiotherapy and chemotherapy. *In* Current Radio-Oncology (Karcher KH, Kogelnik HD, Reinartz G, eds), vol II. New York: Raven Press, 1982, pp 387–391.

(*9*) ARRIAGADA R, LE CHEVALIER T, BALDEYROU P, et al: Alternating radiotherapy and chemotherapy schedules in small cell lung cancer, limited disease. Int J Radiat Oncol Biol Phys 11:1461–1467, 1985.

(*10*) GOLDIE JH, COLDMAN AJ: A mathematic model for relating the drug sensitivity of tumors to their spontaneous mutation rate. Cancer Treat Rep 63:1727–1733, 1979.

(*11*) LOONEY WB, RITENOUR ER, HOPKINS HA: Solid tumor models for the assessment of different treatment modalities. XVI. Sequential combined modality (cyclophosphamide-radiation) therapy. Cancer 47:860–869, 1981.

(*12*) CATANE R, LICHTER A, LEE YJ, et al: Small cell lung cancer: Analysis of treatment factors contributing to prolonged survival. Cancer 48:1936–1943, 1981.

(*13*) GLATSTEIN E: Small cell cancer of the lung: Therapeutic challenge for the 1980s. *In* Recent Advances in Clinical Oncology (Williams CJ, Whitehouse JMA, eds), vol 1. Edinburgh: Churchill Livingstone, 1982, pp 295–303.

(*14*) MINNA JD, BUNN PA JR, CARNEY DN, et al: Experience of the National Cancer Institute (USA) in the treatment and biology of small cell lung cancer. Bull Cancer (Paris) 69:83–93, 1982.

(*15*) PHILLIPS TL, FU KK: The interaction of drug and radiation effects on normal tissues. Int J Radiat Oncol Biol Phys 4:59–64, 1978.

(*16*) LE CHEVALIER T, ARRIAGADA R, PICO JL, et al: Chimiothérapie et radiothérapie séquentielles dans les carcinomes anaplasiques bronchiques à petites cellules. Résultats préliminaires. Bull Cancer (Paris) 69:98–101, 1982.

(*17*) PICO JL, HAYAT M, ARRIAGADA R, et al: High dose combination chemotherapy with autologous bone marrow transplantation in small cell lung cancer. Presented at the Second Workshop on Small Cell Lung Cancer, Edinburgh, 1984.

(*18*) WORLD HEALTH ORGANIZATION: WHO Handbook for Reporting the Results of Cancer Treatment. WHO Offset Publ No. 48. Geneva: World Health Organization, 1979.

(*19*) COX DR: Regression models and life tables. J R Stat Soc 34:187–220, 1972.

(*20*) LE CHEVALIER T, ARRIAGADA R, DE THÉ H, et al: Toxicity of first chemotherapy cycle as a major prognostic factor in survival of patients with limited small cell lung cancer (SCLC). Proc ASCO 5:181, 1986.

(*21*) LE CHEVALIER T, DE THÉ H, ARRIAGADA R, et al: Prognostic value of initial chemotherapy drug doses in patients with small cell lung cancer. Cancer Chemother Pharmacol 18(suppl 1):A43, 1986.

(*22*) AISNER J, ALBERTO P, BITRAN J, et al: Role of chemotherapy in small cell lung cancer: A consensus report of the International Association for the Study of Lung Cancer workshop. Cancer Treat Rep 67:37–43, 1983.

(*23*) HANSEN HH: Management of small-cell anaplastic carcinoma, 1980–1982. *In* Lung Cancer 1982 (Ishikawa M, ed). Amsterdam: Excerpta Medica, International Congress Ser No. 569, 1982, pp 31–54.

(*24*) IHDE DC, BUNN PA: Chemotherapy of small cell bronchogenic carcinoma. *In* Recent Advances in Clinical Oncology (Williams CJ, Whitehouse JMA, eds), vol 1. Edinburgh: Churchill Livingstone, 1982, pp 305–323.

(*25*) SALAZAR OM, CREECH R: The state of the art: Toward defining the role of radiation therapy in the management of small cell bronchogenic carcinoma. Int J Radiat Oncol Biol Phys 6:1103–1117, 1980.

(*26*) BUNN P, IHDE D, LICHTER R, et al: Randomized trial of combination chemotherapy (CT) with or without chest radiotherapy (RT) in limited stage small cell lung cancer (SCLC). Presented at the Fourth World Conference on Lung Cancer, Toronto, 1985.

(*27*) PEREZ CA, EINHORN L, OLDHAM RK, et al: Randomized trial of radiotherapy to the thorax in limited small-cell carcinoma of the lung treated with multiagent chemotherapy and elective brain irradiation: A preliminary report. J Clin Oncol 2:1200–1208, 1984.

(*28*) IHDE D, BROOKS B, SEIFTER E, et al: Pulmonary toxicity (PT) with combined modality therapy for limited stage small cell lung cancer (SCLC). Presented at the Fourth World Conference on Lung Cancer, Toronto, 1985.

Simultaneous Therapy With High-dose Cisplatin and Radiation for Unresectable Squamous Cell Cancer of the Head and Neck: A Phase I-II Study [1]

Richard Wheeler,* Merle Salter, Suzanne Stephens, Irma Hardy, Glenn Peters, Marshall Urist, and William Maddox [2]

ABSTRACT—Studies using simultaneous radiation therapy and conventional doses of cisplatin have suggested improvement in local control and patient survival. This study was undertaken to determine toxicity and patient tolerance to concomitant high-dose cisplatin (40 mg/m^2 per day × 5) and radiation (60 Gy in 6 wk ± 10-Gy boost to residual tumor). Seventeen patients with advanced, inoperable squamous cell cancer primary tumor in the head and neck were treated (15 males and 2 females; median age, 57 yr). Cisplatin was started on day 1 of radiation therapy and repeated every 28 days for three cycles. Normal saline infusion (250 ml/hr) was started 12 hours prior to the first dose and continued 12 hours after the fifth dose. The daily dose of cisplatin was dissolved in 250 ml of 3% NaCl and given over 30 minutes. The cisplatin dose for subsequent cycles was reduced 10 mg/m^2 per day only for a nadir granulocyte count $<500/\text{mm}^3$ or fever >101° F during leukopenia. Of the 17 patients who started therapy, 15 have completed therapy; 1 patient died after one cycle, and 1 died after two cycles. Eleven patients received three cycles of cisplatin, and 10 patients required one dose reduction (6 at course 2 and 4 at course 3). Seven possible infections were successfully treated. Grade 2 neuropathy occurred in 3 patients, and renal toxicity greater than grade 1 occurred in 1 patient. Additional toxic effects were median WBC count nadir of $1.8 \times 10^3/\text{mm}^3$, platelet count nadir of $128 \times 10^3/\text{mm}^3$, hemoglobin nadir of 9.8 g/dl, and median weight loss of 5%. The median total dose of cisplatin administered was 500 mg/m^2. Radiotherapy was interrupted for acute radiation toxicity in 4 patients. All of the 15 patients who have completed therapy have had clinical resolution of tumor, and 5 have relapsed with distant metastases. Median survival for 12 of the 17 patients who were treated is >9 months. High-dose cisplatin plus radiation therapy is an aggressive but manageable treatment program for inoperable head and neck cancer, with a high initial rate of complete response and no apparent increase in acute radiation toxicity.—NCI Monogr 6:339–341, 1988.

Patients with inoperable, locoregional squamous cell cancer primary in the head and neck are routinely treated with radiation therapy. However, the overall survival is low, and the majority of patients die of locoregional tumor involvement (*1*). Laboratory studies have demonstrated that cisplatin is a potent radiation sensitizer (*2,3*). Preliminary clinical trials have suggested that simultaneous irradiation and conventional-dose cisplatin may improve local control and patient survival without a marked increase in acute radiation toxicity (*4,5*). Potentiation of radiation by cisplatin in experimental systems is dose dependent (*3*), and high-dose cisplatin can be administered with hypertonic saline hydration (*6*). This study was undertaken to determine the toxicity and patient tolerance to simultaneous conventional fractionated radiation therapy and high-dose cisplatin in patients with advanced, previously untreated squamous cell cancer of the head and neck.

PATIENTS AND METHODS

From April 1985 through June 1986, 15 males and 2 females with stage III (5 patients) or IV (12 patients) previously untreated squamous cell cancer originating in the head and neck agreed to participate in this combined modality study. All patients were considered to be inoperable candidates for primary radiation therapy. The median age was 57 years (range, 42–68). Additional eligibility criteria were WBC count $\geq 4{,}000/\text{mm}^3$, platelet count $\geq 100{,}000/\text{mm}^3$, bilirubin <1.5 mg/dl, creatinine clearance ≥ 60 ml/minute, Karnofsky performance status $\geq 60\%$, and no distant metastases. This study was approved by the Institutional Review Board of the University of Alabama at Birmingham and the Subcommittee on Human Studies of the Birmingham Veterans Administration Medical Center; all patients gave informed consent.

Radiation therapy (180 cGy/day on Monday through Friday; total dose, 60 Gy) and cisplatin (40 mg/m^2 per day × 5) were both started on day 1. Cisplatin was administered 1–4 hours after the daily radiation treatment. The initial field size used to encompass the primary tumor provided a 2-cm margin to all visible and palpable disease. When neck nodes were present, the fields included the first-echelon lymph nodes as well as the inferior jugular chain nodes and the upper mediastinum. A 1,000-cGy boost to the primary site was delivered in 9 patients who had persistent tumor after receiving 60 Gy. Radiation therapy was delayed for grade 3 or worse skin or mucous membrane toxicity (Radiation Therapy Oncology Group criteria) but was not routinely interrupted for myelosuppression.

Cisplatin courses were repeated every 28 days for a total

---

[1] Supported by Public Health Service grant CA-13148 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and the Clinical Studies Unit, Comprehensive Cancer Center of Alabama.

[2] Departments of Radiation Oncology (*M. Salter*), Surgery (*G. Peters, M. Urist,* and *W. Maddox*), and Medicine (*R. Wheeler, S. Stephens,* and *I. Hardy*), University of Alabama at Birmingham and Veterans Administration Medical Center.

* *Reprint requests:* Richard H. Wheeler, M.D., Department of Medicine, Comprehensive Cancer Center, University of Alabama at Birmingham, 211 Wallace Tumor Institute, University Station, Birmingham, AL 35294.

TABLE 1.—Hematologic and liver toxicity and weight loss

Cycle No.	No. of patients	Median nadir WBC count (× 10^3/mm^3)	Median nadir Platelet count (× 10^3/mm^3)	Median nadir Hemoglobin (g/dl)	Creatinine clearance (ml/min)	Weight loss (%)
0	17	9.2 (6.1–15)	395 (203–623)	13.8 (9.4–16.3)	96 (64–149)	—
1	17	1.8 (0.1–3.3)	165 (18–545)	9.8 (7.6–12.5)	91 (26–140)	4.1 (0–14)
2	14	2.0 (0.4–4.3)	128 (34–467)	10.2 (7.4–12.9)	76 (28–152)	5.0 (0–17)
3	11	2.2 (1.7–4.3)	129 (39–335)	10.6 (9.1–12.6)	68 (57–78)	5.2 (0–19)

of three cycles. A course was delayed until hematologic recovery if the WBC count was <4,000/mm^3 or if the platelet count was <100,000/mm^3 on day 28. The cisplatin dose was decreased by 10 mg/m^2 per day only if the granulocyte count nadir was <500/mm^3 or if the patient became febrile while leukopenic. From 12 hours prior to the first dose of cisplatin through 12 hours following the last dose, the patient received 250 ml/hour of normal saline containing KCl (10 mEq/liter). Each cisplatin dose was dissolved in 250 ml of hypertonic (3%) saline and administered over 30 minutes.

At 4–6 weeks after completion of therapy, patients were independently evaluated in the Departments of Surgery, Radiation Oncology, and Medical Oncology. When it was appropriate, endoscopy, biopsy, and computerized tomography scans were repeated. Determination of response was based on the clinical evaluation only, but all appropriate modalities were utilized for follow-up. A complete response 1 month after the completion of active therapy was defined as no visible or palpable evidence of persistent neoplasm on clinical examination.

RESULTS

Of the 17 patients who started therapy, 15 completed therapy and 2 died during treatment. One patient had marked tumor regression following the first week of therapy but developed abscesses in the involved neck nodes that dissected into the mediastinum and required open surgical drainage. Abscess formation occurred prior to the onset of leukopenia, but the patient did not develop clinical sepsis. Despite aggressive therapy of the abscess, the patient did not recover sufficiently to resume therapy. The second patient also responded dramatically with marked tumor regression and improved performance status. Ten days following the second course of cisplatin, the patient died in his sleep of unknown causes. Serum electrolyte levels and CBC 24 hours prior to death were within normal limits. These patients are considered evaluable for survival and toxicity but not response.

Eleven patients received three courses of cisplatin as planned. In addition to the 2 patients who died, 2 others refused cisplatin after one and two courses. One patient developed ascites secondary to alcoholic cirrhosis after one course of therapy and could not tolerate the necessary hydration. The other patient developed pulmonary edema during hydration for the second cycle of cisplatin. This course was halted after two doses, and a third cycle was not attempted. These patients are considered fully evaluable. The median cumulative dose of cisplatin was 500 mg/m^2 (mean, 420) for all 17 patients and 550 mg/m^2 for the 11 patients who received three courses. Only 1 patient tolerated 200 mg/m^2 for three cycles. All other patients required one dose reduction at the second or third course. Nine of these patients had a 1-week delay before the next course of cisplatin was given.

The systemic and local toxic effects encountered in this study are summarized in tables 1 and 2. Hematologic toxicity was the major toxic effect observed. Seven patients required hospitalization for antibiotic therapy during the WBC count nadir. All patients fully recovered, and only 1 individual had an infectious organism cultured. There were no overt bleeding episodes encountered during thrombocytopenia. The decrease in serum hemoglobin was attributed to cisplatin-induced anemia. Four patients developed neurotoxicity characterized by extremity paresthesias, with mild grip weakness in 3. Ototoxicity was primarily high-frequency hearing loss that worsened with each course of treatment. Three patients had a syncopal episode 3–5 days following the first course of cisplatin; 2 of these patients had evidence of neurotoxicity. This did not recur in 2 patients but did occur after the second course in 1 patient. An etiology for these episodes was not established. The creatinine clearance decreased an average of 28% ± 16% SD in patients who received three courses of cisplatin. However, dose reduction for a clearance <50 ml/minute was necessary in only 1 patient. Nausea and vomiting were well controlled using dexamethasone, lorazepam, and metoclopramide. All patients had fewer than three episodes of emesis. Patients lost a median of 5.2% of initial body weight during treatment.

Acute radiation mucositis and dermatitis were graded using Radiation Therapy Oncology Group criteria. Four patients had radiation therapy interrupted for 5–20 days (median, 9) to allow recovery from toxicity. Two patients had a 9- to 10-day delay during myelosuppression accompanied by fever. Another patient had a delay during acute exacerbation of cirrhosis with ascites, until there was medical stability. Overall, the degree of acute radiation toxicity observed was not different from that expected from radiation therapy alone; a median grade 2 (range, 0–3) skin and mucosal toxicity was observed.

TABLE 2.—Other toxic effects

Toxic effect	No. of patients ($n=17$)	No. of courses ($n=42$)
Infection	7	7
Transfusion	4	4
Neuropathy	4	
Syncope	3	4
Congestive heart failure	1	1

The therapeutic results in the 17 patients treated are summarized in table 3. Patients 15 and 16 died during therapy and are not evaluable for response or site of disease progression. All 15 evaluable patients had clinical resolution of the primary tumor and involved lymph nodes. Five patients had follow-up blind biopsies, and 1 patient underwent a radical neck dissection. One month after completing treatment, three biopsies were negative and the radical neck specimen showed no viable tumor. In 2 patients, isolated tumor cells were noted in the submucosa. However, neither patient has shown evidence of local tumor growth at >8 and >17 months of follow-up. Although the duration of follow-up is short, there have been no instances of locoregional disease progression to date. Five patients have relapsed with distant metastases (bone, 3; liver, 1; and skin, 2) at a median of 7 months after beginning therapy. Median survival with 12 patients still alive is >9 months.

DISCUSSION

Patients with advanced inoperable squamous cell cancer of the head and neck have a poor prognosis. Efforts to use sequential chemotherapy plus irradiation with or without surgery as primary management have generally not been effective (*7,8*), and studies using simultaneous radiation plus methotrexate have not yielded improved results (*9*). In addition, the concomitant use of radiation with bleomycin, methotrexate, or 5-fluorouracil can produce increased normal tissue toxicity that limits the dose of drug or radiation that can be delivered (*1,9*). Cisplatin is an active drug in head and neck cancer (*10*) and a potent radiation sensitizer in experimental systems (*2,3*). To maximize the dose-dependent antineoplastic and radiopotentiation effects, we have combined high-dose cisplatin with conventional fractionated radiation therapy in this phase I–II trial.

TABLE 3.—Therapeutic results

Patient No.	Site	Stage	Time to disease progression (mo)[a]		Survival (mo)
			Local	Distant	
1	Base of tongue	T3 N0	17+	17+	17+
2	Hypopharynx	T3 N3b	16+	16+	16+
3	Tonsil	T4 N0	13+	8	13
4	Base of tongue	T4 N3b	15+	15+	15+
5	Oropharynx	T3 N1	13+	13+	13+
6	Base of tongue	T3 N0	12+	12+	12+
7	Oropharynx	T3 N2b	11+	11	11+
8	Maxillary sinus	T3 N2a	11+	7	11+
9	Tonsil	T3 N2b	9+	9+	9+
10	Nasopharynx	T4 N1	8+	8+	8+
11	Unknown	TX N2b	8+	8+	8+
12	Nasopharynx	T3 N3b	8+	7	8
13	Hypopharynx	T3 N0	4+	4	4
14	Hypopharynx	T3 N3b	5+	5+	5+
15	Base of tongue	T4 N3a	NE	NE	2
16	Tonsil	T4 N3b	NE	NE	1.5
17	Oropharynx	T3 N1	3+	3+	3+

[a] NE = not evaluable.

Despite the high dose of cisplatin administered, there was no appreciable increase in acute radiation toxicity. The major toxic effect observed was myelosuppression, principally leukopenia. Seven patients required antibiotic therapy for fever >101° F. However, all patients were successfully treated, and there were no deaths directly resulting from drug or radiation toxicity. Thrombocytopenia, neuropathy, anemia, and high-frequency hearing loss were observed but did not compromise patient treatment or survival.

Conclusions about therapeutic efficacy are preliminary since the number of patients treated is small and the follow-up short. However, the fact that all 15 patients who completed therapy have attained complete clinical remission is encouraging, and the absence of disease progression within the irradiated field in any patient suggests an improved rate of local control. Five patients have relapsed to date, all in distant metastatic sites.

In summary, therapy with simultaneous high-dose cisplatin and radiation is an aggressive treatment program that can be successfully delivered with careful patient monitoring and supportive care and may improve local control in patients with advanced head and neck cancer. Additional clinical trials and, ultimately, a randomized controlled evaluation will be necessary to fully define any improvement in therapeutic efficacy.

REFERENCES

(*1*) MILLION RR, CASSISI NJ, WITTES RE: Cancer of the head and neck. *In* Cancer: Principles & Practice of Oncology (DeVita VT Jr, Hellman S, Rosenberg SA, eds), 2nd ed. Philadelphia: Lippincott, 1985, pp 407–506.

(*2*) KYRIAZIS AP, YAGODA A, KEREIAKES JG, et al: Experimental studies on the radiation-modifying effect of *cis*-diamminedichloroplatinum II (DDP) in human bladder transitional cell carcinomas grown in nude mice. Cancer 52:452–457, 1983.

(*3*) DOUPLE EB, RICHMOND RC: Enhancement of the potentiation of radiotherapy by platinum drugs in a mouse tumor. Int J Radiat Oncol Biol Phys 8:501–503, 1982.

(*4*) HASELOW RE, ADAMS GS, OKEN MM, et al: Simultaneous cisplatinum and radiation therapy for locally advanced unresectable head and neck cancer. Proc ASCO 1:201, 1982.

(*5*) AL-SARRAF M, MARCIAL V, MOWRY P, et al: Superior local control with combination of high dose cisplatinum and radiotherapy. An RTOG study. Proc Am Assoc Cancer Res 26:169, 1985.

(*6*) OZOLS RF, CORDEN BJ, JACOB J, et al: High dose cisplatin in hypertonic saline. Ann Intern Med 100:19–24, 1984.

(*7*) VOGL SE, LERNER H, KAPLAN BH, et al: Failure of effective initial chemotherapy to modify the course of stage IV (M0) squamous cancer of the head and neck. Cancer 50:840–844, 1982.

(*8*) PETROVICH Z, BLOCK J, KUISK H, et al: A randomized comparison of radiotherapy with a radiotherapy chemotherapy combination in stage IV carcinoma of the head and neck. Cancer 47:2259–2264, 1981.

(*9*) BERTINO JR, CAPIZZI RL: The role of chemotherapy in the management of cancer of the head and neck: A review. Cancer 36:752–758, 1975.

(*10*) LOEHREZ PJ, EINHORN LH: Cisplatin. Ann Intern Med 100:704–713, 1984.

Concomitant Therapy With Infusion of Cisplatin and 5-Fluorouracil Plus Radiation in Head and Neck Cancer

Samuel G. Taylor IV,* Anantha K. Murthy, John Showel, David D. Caldarelli, James C. Hutchinson, Jr., and Lauren D. Holinger[1]

ABSTRACT—We have combined cisplatin, 5-fluorouracil infusion, and radiation in an every-other-week schedule in a phase I-II study of 44 patients with head and neck cancer to assess toxicity and response. Ten patients were treated palliatively with 2 to 6 cycles of therapy, and 34 were treated curatively with a planned 7 cycles. Of 34 patients treated curatively, all were initially controlled. Three died during treatment (1 myocardial infarction, 1 bowel perforation, and 1 renal failure after aminoglycoside antibiotics). Four patients have had regional recurrences, 7 failed at distant sites (follow-up 2 to 5 yr). Thirty-three percent of 20 patients with complete clinical disappearance of all evidence of their cancer have had a recurrence, as have 38% of 14 ($P>.1$) with some residual abnormalities (partial responders) following treatment. All failures were in the 25 patients with T4 and/or N3 disease. None of the 9 patients with lesser stage IV or stage III disease who were followed for 24 months or more had recurrences. Eighteen patients (53%) survive with a projected 3-year survival of 63% (95% confidence interval 47% to 77%). Nine (27%) have died of disease, 1 (3%) died of a second primary in the head and neck, 3 (9%) of intercurrent disease at 15 to 45 months, and 3 (9%) during treatment. Of the 10 patients treated palliatively, 1 died during treatment with hepatic failure, 6 had complete responses, and 2 had partial responses. Median duration of response was 8 months, with 2 continuing in disease control for 50 and 40 months. This combination, which has achieved very promising regional control in this pilot study despite limited surgical intervention, warrants controlled clinical testing.—NCI Monogr 6:343–345, 1988.

Because of the high rate of regional failure in patients with advanced head and neck cancer treated with surgery and radiation and because of the mutilation necessary from radical surgical excision of these cancers, several investigators have used chemotherapy prior to local therapy to improve cure rates and to enable surgical conservation. Neoadjuvant chemotherapy causes high response rates, but randomized studies have failed to show survival benefit (*1*). We have combined chemotherapy with radiation therapy to take advantage of the radiation-potentiating properties of cisplatin and 5-fluorouracil, which together have achieved the highest response rate in head and neck cancer (*2*). We adopted the regimen of Byfield et al. (*3*) of every-other-week treatment with both chemotherapy and radiation, with a 9-day recovery between cycles. This schedule allowed almost full drug dosage and optimal concomitant drug and radiation exposure. In our preliminary report (*4*), we also stressed that our few experiences with conventional radiation fractions, without split course, and this chemotherapy led to severe mucosal toxicity and very limited drug administration. Because of the experimental nature of this study, we treated patients with very advanced head and neck cancer, thought to be incurable with conventional approaches, and only accepted less advanced stage IV and selected stage III patients as the study progressed.

PATIENTS AND METHODS

Patients were entered into this pilot study between July 1981 and June 1984. Initial patient selection included only those patients with advanced stage IV disease or recurrence, who were deemed incurable by the radiation therapist. With increasing experience and confidence in the regimen, we considered for entry to the study patients with earlier stage IV disease and selected patients with stage III hypopharynx or tongue primaries, as a treatment alternative to radical surgery. Age was not a selection factor. A performance status of 4 and prior radiation therapy to a dose in excess of 50 Gy in the previous 2 years excluded patients from entry.

Treatment included 60 mg cisplatin/m^2 on day 1, followed by an infusion of 800 mg 5-fluorouracil/m^2 over 24 hours on days 1–5 (120 hr), and radiation therapy of 2 Gy/day on days 1–5. All treatment was stopped for 9 days, and the cycle was then repeated for a total of 7 cycles (70 Gy). Patients who had received prior radiation received 3 to 4 cycles of combined treatment. Radiation techniques and chemotherapy dose modifications have been described (*4*).

Surgery was done following chemotherapy and radiotherapy, at the discretion of the surgeon, in 11 patients in the curative group. Seven had a radical neck dissection only, 1 a partial glossectomy, and 1 a laryngectomy combined with a neck dissection. Two required laryngectomies for repeated aspiration in 1 and recurrent obstructing edema in the second. One of the patients who had had an elective neck dissection subsequently had a recurrence in the larynx and underwent a successful salvage laryngectomy. Two additional patients had biopsy for histologic documentation of response.

Determination of response was made 6 weeks after completion of therapy or just prior to surgery. Complete response included complete disappearance of all known disease by clinical examination or normalization of x-rays and computerized tomography scans for inaccessible sites. Partial response indicated a 50% reduction in the sum of the products of the longest diameter and its perpendicular for each measurable lesion or an estimated 50% decrease in unilateral measurements of lesions that could be evaluated. Time to relapse and time to death were taken from the start of therapy to the event.

---

[1] Department of Medicine, Section of Medical Oncology (*S. G. Taylor IV* and *J. Showel*), Department of Radiation Therapy (*A. K. Murthy*), and Department of Otolaryngology (*D. D. Caldarelli, J. C. Hutchinson, Jr.,* and *L. D. Holinger*), Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL.

* *Reprint requests:* Samuel G. Taylor IV, M.D., Section of Medical Oncology, Department of Medicine, Rush-Presbyterian-St. Luke's Medical Center, Suite 830, 1725 W. Harrison St., Chicago, IL 60612.

RESULTS

Forty-four patients accepted the study terms and were entered. Thirty-four of these had no distant metastases and could receive full-dose radiation therapy, despite prior therapy in 6 (curative group). Five patients had had prior radiation, which limited the number of their combined cycles to 3 or 4. Three patients presented with distant metastases and uncontrolled regional disease, and 2 had both recurrence following prior radiation and distant metastases (palliative group). Patient characteristics are detailed in table 1. Age ranged from 39 to 83 years (median 62). Twelve (27%) were females. Five (11%) patients had well-differentiated cancers, 20 (45%) moderately well-differentiated, and 18 (41%) poorly differentiated ones. Follow-up ranged from 24 to 60 months (median 36 mo).

Knowing the number of courses received and the full dosage the protocol prescribed, we calculated the percentage of ideal dosage for 5-fluorouracil and cisplatin actually received per cycle. A median of 85% of 5-fluorouracil and 94% of cisplatin was given. In the curative group, patients who relapsed had received significantly less 5-fluorouracil than did those continuing disease free (average 69% vs. 89%; $P<.01$), whereas no such difference existed for cisplatin dosage.

The most frequent toxicity was weight loss, which was more than 10% of body weight in 55% of the patients. This in turn was due to mucositis, with confluent ulcers developing in 49%, and nausea and vomiting that persisted in 25% beyond 3 days. Loss of taste and xerostomia occurred in virtually all patients during treatment. Myelotoxicity was generally not severe, with only 11% having a leukocyte count below 1,500/mm^3 and 9% having a platelet count below 50,000/m^3. Seven percent developed a serum creatinine greater than 2.0 mg/dl (range 2.2–2.7 mg/dl) during treatment.

Four deaths occurred during treatment. Use of an aminoglycoside antibiotic for aspiration pneumonia 3 weeks after cisplatin treatment caused lethal renal failure, although the highest serum creatinine level prior to the antibiotic had been 2.0 mg/dl. One patient with known cirrhosis of the liver died of hepatic failure after 2 cycles. Another died of a presumed myocardial infarction in the interim period between her 4th and 5th cycles, and 1 died of bowel perforation after 3 cycles. These deaths may have been precipitated by the chemotherapy program given to very fragile patients. We now believe hepatic cirrhosis to be a contraindication to this therapy and avoid administering aminoglycoside antibiotics unless there is a specific life-threatening indication.

TABLE 1.—Patient characteristics

Characteristics	Curative group $n=34$	Palliative group $n=10$
Performance status		
2 or worse	26%	50%
Stage		
III	4	—
IV	24	—
Recurrent	6	5
M1	—	5
Primary site		
Oral cavity	9	2
Oropharynx or hypopharynx	15	4
Larynx	4	3
Nasopharynx	2	1
Other	4	—

Curative Group

All patients responded to therapy, with 59% having a complete response and 41% a partial response. In contrast to most other chemotherapy studies, however, degree of response did not correlate with subsequent recurrence. Six of 18 complete responders and 5 of 13 partial responders who completed treatment have subsequently relapsed. Of 13 patients who had surgery or biopsy, 5 of 6 clinically assessed complete responders and 3 of 7 partial responders had complete clearance histologically, which indicated that residual clinical abnormalities in patients with such advanced cancers may not necessarily reflect the true disease status.

Eleven patients (32%) have had recurrences (35% of those who completed treatment). Time to recurrence was 5 to 37 months (median 11 mo). Four of these recurrences were in or near the primary site and none were in the neck. The local recurrence rate was thus 12% (81% local relapse-free survival at 26 mo by Kaplan-Meier estimate). Seven failures occurred distantly in lung (1), bone (3), brain (2), or multiple sites (1) without regional failure. Distant metastases comprised 64% of all failures. In addition, 1 patient (3%) had a second primary develop at 21 months, and 3 (9%) have died free of disease at 15 to 45 months during follow-up. All failures were in the 25 patients with T4 and/or N3 disease. None of 9 patients with lesser stage IV or stage III disease have failed.

Eighteen of the 34 patients treated with curative intent survive (53%). Nine (26%) died from relapse, 3 (9%) from other causes during follow-up, and 1 (3%) from a second upper aerodigestive tract primary. The actuarial survival at 1 year was 76%, at 2 years 68%, and at 3 years 63%. At 3 years, the 95% confidence interval for survival was 47% to 77% (*5*).

Palliative Group

Six had complete responses, 3 had partial responses, and the disease of 1 was stable. One partial responder died during treatment of progressive hepatic failure. Duration of response of the remaining responders was 8 months. However, 2 of the 5 patients without distant metastases at the start of treatment remain disease free at 40 and 50 months, respectively. Two other responders failed regionally and 4 failed in distant sites.

DISCUSSION

This pilot study on the use of concomitant radiation and cisplatin and 5-fluorouracil chemotherapy establishes the feasibility of this approach. The treatment required hospitalization of most patients and a central venous access device due to phlebitis from the 5-fluorouracil infusion. Selected patients could receive outpatient infusion pumps for at least part of the treatment. Because no randomized comparison existed, it is difficult for us to quantitate toxicity. Our impression was that mucositis was generally less than that expected from conventional radiation, but weight loss and anorexia were worse and recovery from the local toxicities of loss of taste and tissue edema were longer. Four patients (9%) died during treatment. Although the deaths were not the usual chemotherapy-related deaths, they emphasize the frailty of these patients. Nevertheless, we were able to treat patients up to the age of 80 with this regimen.

This treatment achieved remarkable regional control in this advanced patient population. Only 13% of patients have failed locally (none in the neck) after a minimal follow-up of 2 years. Indeed, the ratio of 1:2 of local to distant failures was the reverse of that expected from results of other studies in head and neck cancer. Even in the palliative group, only 2 patients failed regionally. This represents a substantial palliation in view of the horrible complications that result from failure to control disease regionally. Surprisingly, 2 patients have continued disease free for more than 3 years after treatment following failure of prior surgery and radiation therapy.

Our results differ from induction chemotherapy studies in that we found no correlation between clinical response and subsequent disease-free survival. Only 38% of our partial responders have relapsed in our curative group. Histologic sampling of 7 such patients indicated 3 had no cancer pathologically. Residual neck masses, fibrosis, and bone destruction made clinical assessment of residual tumor unreliable. Unfortunately, repeated biopsy, especially of the hypopharynx and larynx, can risk laryngeal edema, chondritis, and aspiration. We discourage any manipulation of these structures, especially in the first 6 months following therapy.

These results are encouraging enough, we hope, to lead others to investigate this approach critically. Currently, we are comparing this approach with the more conventional one of 3 cycles of induction cisplatin and 5-fluorouracil infusion followed by regional treatment. This study will test the concepts of sequential versus concomitant therapy. Other randomized studies are required for evaluation of the relative efficacy and toxicity of this regimen.

REFERENCES

(*1*) Taylor SG IV: Integration of chemotherapy into the combined modality therapy of head and neck squamous cancer—an hypothesis. Int J Radiat Oncol Biol Phys 13:779–783, 1987.

(*2*) Rooney M, Kish J, Jacobs J, et al: Improved complete response rate and survival in advanced head and neck cancer after three-course induction therapy with 120-hour 5-FU infusion and cisplatin. Cancer 55:1123–1128, 1985.

(*3*) Byfield JE, Sharp TR, Frankel SS, et al: Phase I and II trial of five-day infused 5-fluorouracil and radiation in advanced cancer of the head and neck. J Clin Oncol 2:406–413, 1984.

(*4*) Taylor SG IV, Murthy AK, Showel JL, et al: Improved control in advanced head and neck cancer with simultaneous radiation and cisplatin/5-FU chemotherapy. Cancer Treat Rep 69:933–939, 1985.

(*5*) Simon R: Confidence intervals for reporting results of clinical trials. Ann Intern Med 105:429–435, 1986.

Simultaneous Radiotherapy and Chemotherapy With 5-Fluorouracil and Cisplatin for Locally Confined Squamous Cell Head and Neck Cancer

David J. Adelstein,* Vishwa M. Sharan, A. Scott Earle, Ajit C. Shah, Catherine Vlastou, Chandra D. Haria, Susan G. Carter, Catherine Damm, and John D. Hines[1,2]

ABSTRACT—**Fifty-four patients with previously untreated or minimally treated locally confined (M0) squamous cell carcinoma of the head and neck were treated with chemoradiotherapy employing multiple courses of simultaneous radiation, cisplatin, and a 4-day 5-fluorouracil infusion. Twenty-eight patients subsequently underwent definitive surgery, and 26 were treated without surgical resection. Of the 54 patients, 51 were ultimately rendered disease free by this combined modality protocol. The projected relapse-free survival rate for the entire cohort is 71%, with a median relapse-free survival time >17 months. Thirteen patients who had tumors that were technically operable did not undergo surgery after achieving a complete response to induction chemoradiotherapy. Only 1 of these patients experienced subsequent local failure. Although the treatment-associated mucositis and myelosuppression were significant, this chemoradiotherapeutic protocol offers a significant chance of relapse-free survival for all patients with locally confined disease and merits comparison with more standard treatment approaches.—NCI Monogr 6:347-351, 1988.**

Two of the most frequently employed chemotherapeutic agents for squamous cell carcinomas of the head and neck have been 5-FU and cisplatin. Response rates >85% are seen when these drugs are used in combination in previously untreated patients (*1-3*). However, induction chemotherapy with these or other agents prior to definitive surgery and/or radiation therapy has failed to produce any survival advantage, compared to standard treatment without initial chemotherapy (*2,4-7*).

Both 5-FU and cisplatin also have significant radiosensitizing properties (*8,9*), and the simultaneous use of radiation therapy with one or both has been successfully employed in a number of neoplasms (*10-17*). For head and neck cancer, we theorized that a preoperative dose of radiation considered ineffective when given alone might be of much greater value when given with simultaneous radiosensitizing chemotherapy, especially if the chemotherapy had independent activity. Chemotherapeutic radiosensitization might result in meaningful downstaging after limited radiation therapy while avoiding the complications of subsequent surgery in a heavily irradiated field. Ample precedent for such an approach has been established in squamous cell carcinoma of the anus, using 5-FU and mitomycin (*15*).

Since 1983, a pilot protocol using simultaneous radiotherapy and chemotherapy has been utilized at Cleveland Metropolitan General Hospital for patients with locally confined squamous cell carcinoma of the head and neck. We report our results from the first 55 patients treated with this protocol.

MATERIALS AND METHODS

All patients with histologically documented, measurable squamous cell carcinoma of the head and neck region, excluding the nasopharynx, without prior treatment or with minimal prior surgery alone, were eligible for study. Pretreatment staging evaluation included a medical history, examination under anesthesia, panendoscopy, barium swallow, computerized tomographic scan of the involved region, and chest x-ray. Radionuclide scans and other staging procedures were obtained only if clinically indicated. On all patients, we performed a CBC; a urinalysis; an ECG; and serum chemistry tests, including urea nitrogen, creatinine, calcium, phosphorus, alkaline phosphatase, SGOT, SGPT, albumin, total protein, bilirubin, and uric acid. All patients underwent a pretreatment dental evaluation with appropriate care.

Patients were staged according to the TNM system of the American Joint Committee on Cancer. Patients with any tumor size (T) or nodal status (N) were eligible; those with metastatic disease (M1) were ineligible. Patients with serum creatinine >2.0 mg/dl, serum bilirubin >2.5 mg/dl, or an unexplained abnormal pretreatment hemogram were excluded. No age restrictions were placed.

This study was approved by the Cleveland Metropolitan General Hospital Committee on Investigation in Humans, and written informed consent was obtained from all patients before the start of therapy. Patient management was provided by a multidisciplinary team including medical, radiation, and nurse oncologists; otolaryngologists; and plastic and oral surgeons.

Treatment was initiated with both radiation therapy and chemotherapy (fig. 1) and was analogous to similar treatment protocols used for squamous cell cancers of the anus

---

ABBREVIATION: 5-FU = 5-fluorouracil.

[1] Department of Medicine (*D. J. Adelstein, S. G. Carter,* and *J. D. Hines*), Department of Radiology (*V. M. Sharan*), Department of Surgery (*A. S. Earle, A. C. Shah, C. Vlastou,* and *C. D. Haria*), and Department of Nursing (*C. Damm*), Cleveland Metropolitan General Hospital and Case Western Reserve University, OH.

[2] We thank Anne L. LaGania for secretarial assistance in manuscript preparation.

* *Reprint requests:* David J. Adelstein, M.D., Division of Hematology-Oncology, Department of Medicine, Cleveland Metropolitan General Hospital, 3395 Scranton Rd., Cleveland, OH 44109.

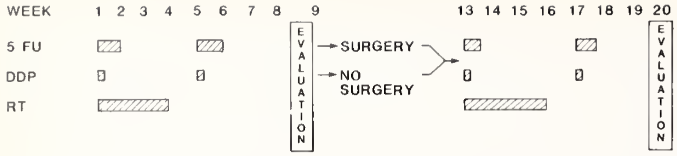


FIGURE 1.—Treatment schema: 5-FU at dose of 1,000 mg/m^2 per day iv by continuous infusion for 4 days; cisplatin (DDP) at 75 mg/m^2 iv on day 1; and radiation therapy (RT) at 3,000 rad in 15 fractions over 3 wk.

and esophagus (*11,15*). Patients were given 3,000 rad of external beam radiation therapy in 15 daily fractions over 3 weeks to the entire tumor and node-bearing region. Radiation was generated by either a cobalt-60 source or a 6-MeV linear accelerator and was administered to midplane without beam modifiers. Concurrent with radiation, patients were given 5-FU (1,000 mg/m^2 per day) for the first 4 days as a continuous iv infusion and cisplatin (75 mg/m^2) iv on day 1. The cisplatin was given after vigorous iv hydration with a furosemide- and mannitol-induced diuresis. Antiemetic agents were given to all patients as necessary. Between weeks 5 and 7, a second identical course of chemotherapy was given but without radiation therapy.

Between 8 and 12 weeks after the initiation of treatment, patients were evaluated for surgery. Operability was defined by the ability to obtain tumor-free margins and a functional reconstruction. Midway during this study, it became apparent that pathological complete remission was possible after preoperative therapy. Because it was difficult to justify extensive head and neck surgery in such patients, surgery became optional for those who had achieved complete remission after preoperative treatment. Initial inoperability did not affect the decisions made after chemoradiotherapy concerning operability or the extent of surgery needed. Surgery consisted of a composite resection of any residual primary tumor, with immediate reconstruction, if possible, and a functional or radical neck dissection.

All patients, whether treated with surgery or not, were then offered continued chemoradiotherapy identical to that already given. An additional 3,000 rad of radiation therapy in 15 daily fractions was given concurrently with a third course of 5-FU and cisplatin. The last 1,000–1,500 rad of this radiation was given to a more limited port that encompassed only the tumor and spared the spinal cord. A fourth course of chemotherapy without radiation therapy completed the planned treatment. Thus, a total of 6,000 rad of radiation therapy and four courses of chemotherapy were given to patients completing all therapy.

Chemotherapy dosage modifications were not made, irrespective of nadir blood counts or duration of myelosuppression. Chemotherapy courses could be delayed a maximum of 3 weeks to allow for full bone marrow and mucosal recovery. If additional delay was considered necessary, the subsequent chemotherapy course was deleted entirely. Permanent deterioration in renal function was considered a criterion for discontinuation of cisplatin.

Patients were evaluated for clinical response after the first two courses of chemotherapy and the first 3,000 rad of radiation. Response was determined by repeating all of the relevant staging procedures. Pathological confirmation of the clinical response was obtained for all surgical and most nonsurgical patients. Clinical response was again evaluated after the completion of all therapy. Patients were subsequently seen every 2 months and clinically assessed for recurrence, with repetition of radiographic procedures only if indicated.

Complete response was defined as the complete disappearance of all radiographic, clinical, and when applicable, pathological evidence of disease. Partial response was defined as any response less than complete but with >50% reduction in the sum of the products of the crossed diameters of all measurable lesions. Patients with less tumor shrinkage were considered nonresponders. Progressive disease was defined as a >25% increase in the sum of the products of the crossed diameters of all measurable lesions or the appearance of locally recurrent or metastatic disease.

Survival curves were constructed using the Kaplan-Meier method. Statistical data were calculated by the Breslow modification of the generalized Wilcoxon test. Survival times were calculated from the date chemotherapy was initiated, and the results were analyzed as of September 1, 1986. The survival times of relapse-free patients who died of unrelated causes are considered censored data at the time of patient death.

RESULTS

Between February 1983 and November 1985, 55 consecutive eligible patients with squamous cell carcinoma of the head and neck were seen at Cleveland Metropolitan General Hospital and were entered in this treatment protocol. There was a single major protocol violation. This patient has been excluded from all analysis, so there are 54 evaluable patients. Fifty of these patients were previously untreated. Four patients had undergone minimal prior surgical excision of the primary lesion, which had subsequently recurred. No patient had previously received radiation therapy or chemotherapy.

TABLE 1.—Patient and tumor characteristics[a]

Characteristic	No. of patients
Sex	
Male	40
Female	14
Race	
White	40
Black	14
Median age in yr (range): 57 (32–77)	
Site	
Oral cavity	21[b]
Oropharynx	20[b]
Hypopharynx	4
Larynx	
Supraglottic	6
Glottic	4
Stage	
I	1
II	6[b]
III	21
IV	27

[a] There were 54 patients and 55 tumors.

[b] Includes 1 patient with 2 synchronous stage II tumors.

The clinical characteristics of the 54 evaluable patients and their tumors are shown in table 1. The extent of the tumor (T) and of nodal involvement (N) is given in table 2. All patients had an Eastern Cooperative Oncology Group performance status of 0–1.

Eleven patients in this cohort had been previously treated for or have subsequently developed a second primary neoplasm. Two patients have had 3 primary tumors each. The second or third neoplasms were generally tobacco related and included 5 squamous cell head and neck cancers, 5 pulmonary neoplasms, 1 bladder cancer, and 4 unrelated malignancies. Two patients presented with 2 simultaneous primary tumors; 1 patient underwent resection of a primary lung cancer, followed by combined modality treatment for the head and neck primary tumor, and the other presented with 2 synchronous but separate head and neck tumors.

The toxicity from this chemoradiotherapeutic approach was significant (table 3), but no deaths from toxicity occurred. Mucositis was almost universal and was best quantitated by the resultant mean weight loss of 8.0 kg (range, 0–23) or 12% of initial body weight. Nephrotoxicity was defined as a >50% increase in the serum creatinine level; it occurred in 17 (31%) of the patients but was completely reversible in all of them. Life-threatening myelosuppression with fever prompted hospitalization of 9 patients (17%), and 9 additional patients required hospitalization because of mucositis.

Five of the 28 surgical patients refused postoperative therapy, and 4 were not treated further due to delayed postoperative recovery. The second and/or fourth courses of chemotherapy alone were not given to 16 additional patients because of patient refusal or protracted hematologic or mucosal recovery. Small weekly doses of methotrexate (20 mg/m^2) were given during radiation therapy to the first 2 patients treated in this study. Methotrexate was not given to any other patients because of our concern about mucositis.

The surgical group included 24 patients who underwent resection of the primary lesion with a node dissection and 4 who underwent lymph node dissection alone after preoperative treatment resulted in complete disappearance of the primary lesion. Surgical complications included delayed healing in 4 patients and a minor wound infection in 2. Late complications for the entire patient cohort included osteoradionecrosis in 1 patient and clinical or biochemical hypothyroidism in 8 patients (15%).

All patients achieved at least partial response to this treatment protocol. The data are analyzed, therefore, in terms of the achievement and maintenance of disease-free status (complete response), as shown in table 4.

TABLE 2.—Extent of tumor and nodal involvement[a]

	No. of patients with—				
	TX	T1	T2	T3	T4
N0		1	6[b]	10	7
N1			6	5	2
N2			3	1	4
N3	2[c]	1		1	6

[a] There were 54 patients and 55 tumors.

[b] Includes 1 patient with 2 synchronous T2 N0 tumors.

[c] Nodal recurrence after limited excision of primary tumor.

TABLE 3.—Chemoradiotherapy toxic effects

Toxic effect	No. of patients (n=54)
Mucositis	53 (98%)
Nausea, vomiting	48 (89%)
Nephrotoxicity	17 (31%)
Neutropenia	
<1,000 cells/mm^3	24 (44%)
With fever	9 (17%)
Thrombocytopenia	
<50,000 cells/mm^3	1 (2%)
Cardiac toxicity	1 (2%)
Postural hypotension	1 (2%)
Mean weight loss: 8.0 kg (range, 0–23)	

Of the 54 patients, 30 (56%) had achieved disease-free status at the time of the first evaluation, after completing the first half of the chemoradiotherapy. Complete response was pathologically confirmed in 19 of these 30 patients. At the end of all treatment, 51 of the 54 patients (94%) were considered disease free. Twelve of these 51 patients have since relapsed, for a total of 15 treatment failures; all but 3 had recurrence of local disease. The 3 patients with metastatic disease had continued local control until death. With a median follow-up of 21 months, the projected Kaplan-Meier relapse-free survival for the entire patient cohort is 71% (fig. 2). The median duration of relapse-free survival is >17 months. Four patients died from unrelated causes while free of their malignancy, resulting in a projected Kaplan-Meier overall survival of 64% and a median overall survival duration >19 months.

Thirteen patients who had technically operable disease and who had achieved complete response at the time of the first evaluation did not undergo surgery. All proceeded to complete the planned chemoradiotherapy. Five of these 13 patients had stage IV disease, 5 had stage III, and 3 had stage II. Four patients would have required laryngectomy had surgery been done at the time of presentation. Only 1 of these 13 patients has subsequently experienced local failure.

The achievement and maintenance of disease-free status was not associated with age, sex, race, site of tumor origin, size of the primary tumor, or the ability to administer all of the planned therapy. No difference in relapse-free survival exists between the surgical and nonsurgical patients (P= .21), although these patient groups are not comparable. Relapse-free survival was also not significantly different between patients with stage I–II, III, and IV disease. Indeed, our projected Kaplan-Meier relapse-free survival for patients with stage IV disease is 62%.

DISCUSSION

The radiosensitizing potential of 5-FU and cisplatin has significant therapeutic promise. A controlled trial in head and neck cancer has previously demonstrated the superiority of definitive treatment with a regimen of radiation plus 5-FU, compared to radiation therapy alone (*10*). Neoadjuvant chemotherapeutic radiosensitization with both of these drugs might theoretically produce a significant tumor

TABLE 4.—Achievement and maintenance of disease-free status

	No. of patients	No. of patients disease free			
		Evaluation 1	After surgery	Evaluation 2	Current status[a]
Surgical group					
Primary resection	24	10[b]	22	22	19
Node dissection only	4	2[b]	4	4	3
Subtotal	28	12	26	26	22
Nonsurgical group					
Inoperable	9	3		8	3
Operable					
Refused surgery	4	2		4	3
Complete response	13	13		13	11
Subtotal	26	18		25	17
Total	54	30 (56%)		51 (94%)	39

[a] Status as of Sept. 1, 1986.
[b] No pathological evidence of disease at surgery.

response after limited doses of radiation therapy, thus allowing for less extensive and more successful surgery, while obviating the surgical complications seen after full-dose radiation treatment. Furthermore, the administration of effective systemic chemotherapy such as 5-FU and cisplatin might diminish the subsequent likelihood of distant metastases.

Complete response (disease-free status) is a necessary prelude to long-term survival and was achieved in 94% of our patients. While patients with less than complete response may experience significant palliation, they are not cured of the neoplasm. Even in our patients with stage IV disease, the projected relapse-free survival is 62%. This is superior to historical data (*4,18*) and stresses the need to consider cure as the treatment goal in all patients with locally confined disease.

The potential for preoperative induction therapy such as this chemoradiotherapeutic protocol to modify subsequent surgical therapy must also be considered (*3,19*). Thirteen patients who had technically operable disease did not undergo surgery after achieving complete response to induction chemoradiotherapy. Similarly, 4 surgical patients underwent only a lymph node dissection after initial chemoradiotherapy resulted in complete disappearance of the primary lesion. This approach may, therefore, spare patients the disfiguring and dysfunctional surgery often necessary for this disease, and it does not appear to compromise relapse-free, long-term survival.

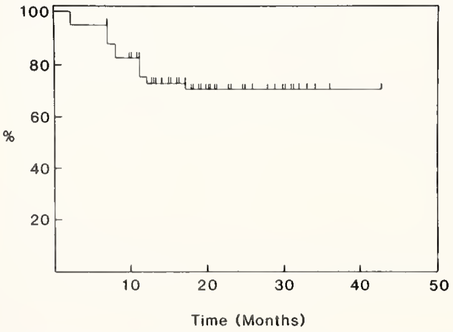


FIGURE 2.—Kaplan-Meier relapse-free survival for entire patient cohort ($N=54$).

It is important to recognize the significant toxicity of this combined modality approach. No treatment deaths occurred, although additional hospitalization was necessary in 18 (33%) of the patients. Life-threatening myelosuppression was noted more frequently than when these chemotherapeutic agents are given alone (*1–3*). Furthermore, the mucositis, although reversible, was often profound, and significant weight loss occurred in most patients. Toxicity such as this can only be justified if the treatment results are significantly better than those of standard therapy. While this study suggests a major improvement in relapse-free, long-term survival, comparative trials with more standard treatment regimens are necessary.

REFERENCES

(*1*) ROONEY M, KISH J, JACOBS J, et al: Improved complete response rate and survival in advanced head and neck cancer after three-course induction therapy with 120-hour 5-FU infusion and cisplatin. Cancer 55:1123–1128, 1985.

(*2*) HAAS C, BYHARDT R, COX J, et al: Randomized study of 5-fluorouracil and *cis*-platinum as initial therapy of locally advanced squamous carcinoma of the head and neck: A preliminary analysis. Proc ASCO 4:143, 1985.

(*3*) JACOBS C, GOFFINET D, FEE W, et al: Chemotherapy as a substitute for surgery in the treatment of advanced operable head and neck cancer: An NCOG pilot. Proc ASCO 4:137, 1985.

(*4*) TAYLOR SG IV, APPELBAUM E, SHOWEL JL, et al: A randomized trial of adjuvant chemotherapy in head and neck cancer. J Clin Oncol 3:672–679, 1985.

(*5*) TANNOCK IF, BROWMAN G: Lack of evidence for a role of chemotherapy in the routine management of locally advanced head and neck cancer. J Clin Oncol 4:1121–1126, 1986.

(*6*) JACOBS C, WOLF GT, MAKUCH RW, et al: Adjuvant chemotherapy for head and neck squamous carcinomas. Proc ASCO 3:182, 1984.

(*7*) SCHULLER DE, WILSON H, HODGSON S, et al: Preoperative reductive chemotherapy for stage III or IV operable epidermoid carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx, phase III: A Southwest Oncology Group study. *In* Proceedings of the International Conference on Head and Neck Cancer. Baltimore: Lancaster Press, 1984, p 48.

(*8*) Vietti T, Eggerding F, Valeriote F: Combined effect of X radiation and 5-fluorouracil on survival of transplanted leukemic cells. J Natl Cancer Inst 47:865–870, 1971.

(*9*) Douple EB, Richmond RC: Radiosensitization of hypoxic tumor cells by *cis*- and *trans*-dichlorodiammineplatinum(II). Int J Radiat Oncol Biol Phys 5:1369–1372, 1979.

(*10*) Lo TCM, Wiley AL, Ansfield FJ, et al: Combined radiation therapy and 5-fluorouracil for advanced squamous cell carcinoma of the oral cavity and oropharynx: A randomized study. Am J Roentgenol 126:229–235, 1976.

(*11*) Leichman L, Steiger Z, Seydel HG, et al: Combined preoperative chemotherapy and radiation therapy for cancer of the esophagus: The Wayne State University, Southwest Oncology Group, and Radiation Therapy Oncology Group experience. Semin Oncol 11:178–185, 1984.

(*12*) Murthy AK, Taylor SG, Showel J, et al: Improved results with simultaneous chemotherapy and radiation in head and neck cancer. Proc ASCO 4:138, 1985.

(*13*) Trybula M, Taylor SG IV, Bonomi P, et al: Preoperative simultaneous cisplatin/5-fluorouracil and radiotherapy in clinical stage III, non-small cell bronchogenic carcinoma. Proc ASCO 4:182, 1985.

(*14*) Byfield JE, Sharp TR, Frankel SS, et al: Phase I and II trial of five-day infused 5-fluorouracil and radiation in advanced cancer of the head and neck. J Clin Oncol 2:406–413, 1984.

(*15*) Leichman L, Nigro N, Vaitkevicius VK, et al: Cancer of the anal canal. Model for preoperative adjuvant combined modality therapy. Am J Med 78:211–215, 1985.

(*16*) Slotman GJ, Cummings FJ, Glicksman AR, et al: Preoperative simultaneously administered *cis*-platinum plus radiation therapy for advanced squamous cell carcinoma of the head and neck. Head Neck Surg 8:159–164, 1986.

(*17*) Kalra J, Cortes E, Chen S, et al: Effective multimodality treatment for advanced epidermoid carcinoma of the female genital tract. J Clin Oncol 3:917–924, 1985.

(*18*) Ucmakli A: The treatment of patients with stage IV squamous cell carcinoma of the head and neck. Otolaryngol Clin North Am 13:529–532, 1980.

(*19*) Pfister D, Bosl GJ, Vikram B, et al: Larynx preservation in patients with advanced head and neck cancer: Neoadjuvant chemotherapy and primary radiation therapy. Proc ASCO 5:140, 1986.

Sequential Administration of Methotrexate, Cisplatin, and 5-Fluorouracil in Multimodal Therapy for Locally Advanced Head and Neck Cancer

Everett E. Vokes,[1,*] William J. Moran,[2] Azhar M. Awan,[1] Richard L. Schilsky,[1] Michael Goldman,[3] Harold G. Sutton,[1] Ralph R. Weichselbaum,[1] and William R. Panje[2,4]

ABSTRACT—Thirty-eight previously untreated patients with locally advanced head and neck cancer received three cycles of induction chemotherapy with methotrexate (120 mg/m^2) followed by cisplatin (100 mg/m^2) and a 5-day continuous infusion of 5-fluorouracil (1,000 mg/m^2 per day). The response rate in 34 evaluable patients was 94%, with a complete response rate of 26%. Thirty-one patients underwent local therapy following induction chemotherapy, and 25 (81%) were rendered free of disease: 14 of 15 treated with surgery and radiotherapy and 11 of 16 treated with radiotherapy alone. At a median follow-up of 11 months, 8 patients have relapsed while the remaining 17 patients continue free of disease. The dose-limiting toxicity of chemotherapy was mucositis resulting in reduction of the 5-fluorouracil dose in 28 patients. This regimen is highly effective in inducing responses in patients with locally advanced head and neck cancer; 81% of the patients who complete local therapy are rendered free of disease with this multimodal approach. Due to short follow-up, the relapse rate, overall survival, and disease-free survival cannot yet be determined.—NCI Monogr 6:353-356, 1988.

In an effort to improve the prognosis of locally advanced head and neck cancer, a number of investigators have added neoadjuvant chemotherapy to traditional therapy with surgery and radiotherapy (*1,2*). Several chemotherapy regimens have been used in this setting and have frequently produced response rates >80% and CR rates as high as 54% (*3-8*). Improvement in long-term survival is suggested when the results of these studies are compared to those in historic controls (*3,5*), but no prospective randomized study has confirmed these findings to date (*9*).

A well-studied neoadjuvant regimen has been the combination of cisplatin and a 5-day continuous infusion of 5-FU as pioneered (*3*) at Wayne State University (Detroit, MI). Since patients achieving CR may have improved long-term survival (*3,4*), we sought to increase the CR rate of the cisplatin–5-FU regimen. Methotrexate, another active single agent in head and neck cancer (*1,2,10*), was added to the combination of cisplatin and 5-FU in a sequential fashion, preceding the 5-FU infusion by 30 hours (*11*). We now report initial results in 38 patients treated with this regimen prior to local therapy.

PATIENTS AND METHODS

Between January 1984 and December 1985, 38 patients were registered in this protocol. All patients had biopsy-proven squamous cell or mucoepidermoid carcinoma of the head and neck or lymphoepithelioma of the nasopharynx. Sequential staging procedures for all patients included a history and physical examination, CBC, routine blood chemistry tests, 24-hour urine creatinine clearance, chest x-ray, barium swallow, bone scan, and computerized tomography scan of the head and neck. The extent of local tumor and regional metastases was further assessed by triple endoscopy. Tumor maps were drawn, and the clinical stage was determined according to the American Joint Committee on Cancer Staging and End-Results Reporting (*12*). Prior to the start of treatment, optimal local therapy was determined.

Criteria for eligibility in the study were previously untreated locally advanced stage III or IV disease; <30% statistical probability of 5-year survival following treatment with surgery and/or radiotherapy alone; performance status ≤2 (Eastern Cooperative Oncology Group criteria); and measurable disease. Signed informed consent was also required.

The treatment plan called for three cycles of induction chemotherapy and evaluation for response according to standard response criteria. Patients with evidence of disease progression while receiving chemotherapy immediately proceeded to local therapy consisting of surgery followed by radiotherapy, except for those who refused surgery or who had unresectable disease. Such patients received radiation therapy only, with curative intent. Three additional cycles of chemotherapy were given after local therapy to patients who achieved response to initial chemotherapy and had no residual disease at completion of local therapy.

Chemotherapy consisted of methotrexate (120 mg/m^2) given on day 1 and cisplatin (100 mg/m^2) given as a 6-hour infusion on day 2, followed immediately by 5-FU (1,000

ABBREVIATIONS: CR = complete remission; 5-FU = 5-fluorouracil; PR = partial remisson; MR = minimal response.

---

[1] Department of Medicine, Joint Section of Hematology-Oncology (*E. E. Vokes* and *R. L. Schilsky*) and Department of Radiation Oncology (*A. M. Awan, H. G. Sutton,* and *R. R. Weichselbaum*), The University of Chicago and Michael Reese Hospital and Medical Center, IL.

[2] Department of Surgery, The University of Chicago, IL.

[3] Department of Surgery, Michael Reese Hospital and Medical Center, Chicago, IL.

[4] We thank Carol M. Guarnieri, Susan M. Whaling, and Barbara A. Knapp for nursing care; Sonia McGee for preparing the manuscript; and Dr. Eric P. Lester for supervising patient care during 1984.

* *Reprint requests:* Everett E. Vokes, M.D., Joint Section of Hematology-Oncology, Department of Medicine, Michael Reese Hospital and Medical Center, Lake Shore Dr. at 31st St., Chicago, IL 60616.

mg/m^2 per day) administered by continuous iv infusion for 5 days. Leucovorin rescue (25 mg/m^2) was given every 6 hours for six doses beginning on day 2. Cycles were repeated every 21 days. Chemotherapy doses were modified according to the toxic effects observed during the previous cycle. Delay of the next cycle by 1 week was required if the WBC count was $\leq$2,500/μl; if the platelet count was $\leq$75,000/μl; or if residual mucositis occurred on day 22. The dose of cisplatin was reduced according to the creatinine clearance and the CBC on day 22. If creatinine clearance was 30–50 ml/minute, 50% of the calculated dose was given; if creatinine clearance was <30 ml/minute, no cisplatin was administered. On day 22, if the WBC count was 2,500–3,500/μl and/or the platelet count was 75,000–100,000/μl, 75% of the calculated dose was administered. The dose of 5-FU was reduced by decreasing the total duration of the infusion based on the results of a CBC on day 22 and the degree of mucositis observed in the previous cycle. If the WBC count was 2,500–3,500/μl and/or the platelet count was 75,000–100,000/μl, 80% of the calculated dose of 5-FU was given. If the patient had grade 2 mucositis with ulcers and compromised food intake, 80% of the calculated dose was given; if the patient had grade 3 mucositis with ulcers and was unable to eat any solid food, 60% of the calculated dose was given. The dose of methotrexate was not modified.

Radiotherapy was delivered by a 4- or 6-mV linear accelerator at daily doses of 180–200 cGy. For gross disease, total doses were 66–80 Gy, using electron or interstitial boosts when appropriate. For postoperative radiotherapy, doses were 60 Gy to areas of original gross disease and 45–60 Gy to areas of potential microscopic disease.

Survival was calculated from the date of enrollment in the protocol, and disease-free survival was calculated from the time CR was achieved. The survival rate was calculated as described by Kaplan and Meier (*13*).

RESULTS

Thirty-eight patients are included in this analysis; they were monitored from January 1984 to August 1986. Patient characteristics are shown in table 1.

Stage III disease was diagnosed in 2 patients, stage IV in 36. Staging according to the TNM classification is shown in figure 1. Most patients had far-advanced primary and nodal disease.

Of 38 patients registered in this protocol, 34 completed induction chemotherapy and are available for response analysis. A CR was defined as complete disappearance of all objective clinical evidence of disease; a PR was defined as an average decrease >50% in the volume of measurable lesions; and an MR was defined as clinically significant average tumor regression of 25%–50%. Nine patients (26%) had clinical CR in all sites; 6 had pathological confirmation of CR at biopsy (5 patients) or surgery (1 patient), and 3 had microscopic residual disease at surgery. Twenty-one patients (62%) had PR, 2 (6%) had MR, and 2 (6%) had no response to induction chemotherapy.

Analysis of our data by nodal stage revealed an overall response rate of 100% for N0–N2 disease and 88% for N3 disease; the CR rate was 60% for stage N0, 50% for stage N1, 33% for stage N2, and 6% for stage N3.

At a median follow-up of 11 months from the time of diagnosis, the median actuarial survival for all 38 patients in this study is 18 months. For patients achieving CR, the median survival has not been reached at 17 months of the longest follow-up; for patients achieving PR or MR, it has not been reached at 31 months; and for the 2 patients who had no response, the median survival is 13 months.

TABLE 1.—Patient characteristics[a]

Characteristic	
No. of patients	38
Sex	
Male	27
Female	11
Age (yr)	
Median	53
Range	15–68
Follow-up from diagnosis (mo)	
Median	11
Range	1–31
Performance status	
Grade 0	15
Grade 1	19
Grade 2	4
Histology	
Squamous cell cancer	35
Lymphoepithelioma	2
Mucoepidermoid cancer	1
Sites of disease	
Oral cavity	6
Nasopharynx	4
Oropharynx	14
Hypopharynx	5
Larynx	4
Paranasal sinuses	4
Salivary gland	1

[a] Unless otherwise specified, values = No. of patients.

Prior to local therapy, 3 patients were lost to follow-up. Thus, 31 of 34 patients received local therapy as planned at the time of diagnosis. Fifteen patients were treated with surgery followed by radiotherapy, and 14 were rendered free of disease. Sixteen patients received radiation therapy only. Two of these patients refused surgery; 5 were considered to have unresectable disease because of the anatomic location of the primary disease and 9 because of the advanced nature of the primary disease. Eleven of these 16 patients were rendered free of disease, and 5 had persistent disease (macroscopic in 4 patients; microscopic in 1 patient). Thus, 25 of 31 patients (81%) who completed induction chemotherapy and subsequent local therapy were rendered free of disease. At the time of this analysis, the median actuarial disease-free interval is 9 months (range, 2–28). Eight patients have developed recurrent disease (locoregional recurrence, 6 patients; distant metastases, 1 patient; and locoregional recurrence and distant metastases, 1 patient). Only 1 patient had achieved CR to induction chemotherapy; 7 had PR or MR. Of the 6 patients who were not rendered free of disease, 2 received no further treatment, and 4 received additional chemotherapy. Four of these patients have died 6–13 months after the initial diagnosis, and 2 are alive with active disease at 13 and 15 months of follow-up.

		Patient Stage				
Stage		N0	N1	N2	N3	Total
I	T1	—	—	—	2	2
II	T2	—	—	—	2	2
III	T3	1	1	6	7	15
IV	T4	4	4	4	7	19
	Total	5	5	10	18	

FIGURE 1.—Stage according to TNM classification.

Of 25 patients eligible for additional chemotherapy, 4 relapsed prior to resumption of chemotherapy and 5 refused further chemotherapy. Of 16 patients who received additional chemotherapy, 5 received less than three cycles because of severe toxicity.

The dose-limiting toxicity observed with 105 cycles of induction chemotherapy was mucositis affecting all but 1 patient in the study (mild to moderate in 24 patients, severe in 11). One of these patients had skin desquamation and severe mucositis, developed septicemia, and died. This patient represents the only treatment-related death in this study. Myelosuppression was mild to moderate. The median nadir WBC count was 3,100/μl (range, 900–5,000); the median nadir hematocrit was 29% (range, 24%–36%); and the median nadir platelet count was 158,000/μl (range, 11,000–390,000). Cisplatin-induced renal dysfunction (serum creatinine >2.0 mg/dl) was observed in 8 patients and was reversible in each case. Cisplatin doses were reduced in 21 cycles (14 patients) by 20%–50%. Reductions of the 5-FU dose of 20%–50% (2.5–4 days of 5-FU infusion) were necessary in 60 cycles (28 patients), usually because of mucositis during the preceding cycle of chemotherapy. Twenty cycles were delayed 1 week because of residual mucositis, and three cycles were delayed because of patient noncompliance.

Generally, the toxic effects of chemotherapy administered after local therapy were more pronounced than those with induction chemotherapy. In 41 cycles administered, mucositis was again the dose-limiting toxicity. It frequently occurred while the patient was still receiving 5-FU and was most pronounced in the previously irradiated field. Myelosuppression was also more pronounced; the median nadir WBC count was 2,400/μl (range, 700–5,200); the median nadir hematocrit was 28% (range, 21%–37%); and the median nadir platelet count was 123,000/μl (range, 37,000–298,000). Dose reduction of cisplatin was necessary in 28 cycles (14 patients), and 5-FU dose reduction was necessary in 36 cycles (15 patients).

DISCUSSION

In this pilot study, we sought to improve the efficacy of available neoadjuvant chemotherapy regimens in head and neck cancer by adding methotrexate in a sequential fashion to the combination of cisplatin and 5-FU.

The regimen was shown to be highly active, but the data do not differ substantially from others reported in the literature (*3–8*), and a higher CR rate than that achieved in this study has been reported for the combination of cisplatin and 5-FU alone (*3*). Thus, the increased toxicity resulting from the addition of methotrexate to cisplatin and 5-FU is not balanced by an improved CR rate. This may in part be due to the far-advanced nodal stage of disease in many patients in this series. Such patients have been shown to be less likely to achieve CR (*3,6*). At the same time, however, it is disappointing that advanced nodal disease was not eradicated more efficiently by adding methotrexate to the cisplatin and 5-FU regimen. Also, the increased toxicity seen with the addition of methotrexate resulted in significant dose reductions of 5-FU. Thus, any possible benefit of methotrexate may have been negated by our inability to administer full doses of 5-FU following methotrexate administration. However, response rate alone does not describe the efficacy of a neoadjuvant regimen; overall survival and disease-free survival achieved after completion of all multimodal therapy are also important. Longer follow-up of our patients and information about overall survival, disease-free survival, and pattern of relapse (locoregional vs. distant) therefore are needed to fully describe this regimen and evaluate its efficacy.

Following induction chemotherapy, local therapy rendered 81% of the patients free of detectable disease. Thus, the great majority of patients had at least short-term benefit from this team effort, which may translate into long-term benefit for a substantial fraction of patients. The need for aggressive local therapy following induction chemotherapy is a well-recognized fact that is under dispute only for patients who achieve pathological CR (*14*). In our series, no modifications were made based on response to chemotherapy, and surgery was included in the treatment plan whenever the disease was considered to be resectable. The need for timely and aggressive local therapy is also demonstrated by the outcome for the 3 patients who were temporarily lost to follow-up after completion of induction chemotherapy. Only 1 of these patients was rendered free of disease with local therapy.

The toxic effects of the regimen are well defined through its use in 105 cycles of induction chemotherapy and 41 cycles of adjuvant chemotherapy. The most significant toxic effect clinically was mucositis, which necessitated frequent dose reductions of 5-FU and thus may have offset the possible benefit of the added methotrexate. The other toxic effects described were mild to moderate myelosuppression and transient renal impairment. Overall, the toxic effects of this regimen, although manageable with close patient supervision, are more severe than those described for cisplatin and 5-FU alone (*3*).

While we are impressed with the efficacy of this regimen in inducing remissions in this group of patients with far-advanced disease, it is not clearly superior to other available regimens. Thus, its use cannot be recommended at the present time. Longer follow-up to fully evaluate its impact on overall survival and disease-free survival is needed.

REFERENCES

(*1*) MEAD GM, JACOBS C: Changing role of chemotherapy in treatment of head and neck cancer. Am J Med 73:582–595, 1982.

(*2*) Hong WK, Bromer R: Chemotherapy in head and neck cancer. N Engl J Med 308:75–79, 1983.

(*3*) Rooney M, Kish J, Jacobs J, et al: Improved complete response rate and survival in advanced head and neck cancer after three course induction therapy with 120-hour 5-FU infusion and cisplatin. Cancer 55:1123–1128, 1985.

(*4*) Ervin TJ, Clark JR, Weichselbaum RR, et al: An analysis of induction and adjuvant chemotherapy in the multidisciplinary treatment of squamous-cell carcinoma of the head and neck. J Clin Oncol 5:10–20, 1987.

(*5*) Spaulding M, Ziegler P, Sundquist N, et al: Induction therapy in head and neck cancer. A comparison of two regimens. Cancer 57:1110–1114, 1986.

(*6*) Amrein PC, Fingert H, Weitzman SA: Cisplatin-vincristine-bleomycin therapy in squamous cell carcinoma of the head and neck. J Clin Oncol 1:421–427, 1983.

(*7*) Kies MS, Pecaro BC, Gordon LI, et al: Preoperative combination chemotherapy for advanced stage head and neck cancer: Promising early results. Am J Surg 148: 367–371, 1984.

(*8*) Schuller DE, Wilson HE, Smith RE, et al: Preoperative reductive chemotherapy for locally advanced carcinoma of the oral cavity, oropharynx, and hypopharynx. Cancer 51:15–19, 1983.

(*9*) Tannock IF, Browman G: Lack of evidence for a role of chemotherapy in the routine management of locally advanced head and neck cancer. J Clin Oncol 4:1121–1126, 1986.

(*10*) Bertino JR, Boston B, Capizzi RL: The role of chemotherapy in the management of cancer of the head and neck: A review. Cancer 36:752–757, 1975.

(*11*) Cadman E, Heimer R, Davis L: Enhanced 5-fluorouracil nucleotide formation after methotrexate administration: Explanation for drug synergism. Science 205:1135–1137, 1979.

(*12*) American Joint Committee on Cancer Staging and End-Results Reporting: Manual for Staging of Cancer. Chicago: American Joint Committee on Cancer Staging and End-Results Reporting, 1978.

(*13*) Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481, 1958.

(*14*) Jacobs C, Goffinet D, Fee W, et al: Chemotherapy as a substitute for surgery in the treatment of advanced operable head and neck cancer: An NCOG pilot. Proc ASCO 4:137, 1985.

Chemotherapy With Low Doses of Radiation Followed by Definitive Radiotherapy for Advanced Unresectable Carcinoma of the Head and Neck

M. Bolla,[1,*] J. Borgel,[1] A. Guenoun,[1] C. Junien-Lavillauroy,[1] C. Dionet,[2] and C. Vrousos[1]

ABSTRACT—Thirty-six patients with advanced unresectable carcinoma of the head and neck were treated with a combination of three courses of chemotherapy and low doses of radiation, followed after 3 weeks by definitive irradiation. Each course was repeated every 3 weeks with the following sequence. Cisplatin (20 mg/m^2) was given in a 20-minute infusion, followed by a 2-hour infusion of 5-fluorouracil (400 mg/m^2), on days 1, 2, 5, and 6. Low doses of radiation were given on days 3 and 4, followed by a 2-hour infusion of 5-fluorouracil (400 mg/m^2) with a dose of 3 Gy on the target volume. For definitive irradiation, a total dose of 60 Gy was delivered in 30 fractions within 6 weeks. The complete response rate reached 30%, and the partial response rate was 30%. With a median follow-up of 11 months, median overall survival was 10 months; median survival was 21 months for patients with complete response, 9 months for patients with partial response, and 6 months for those with no response (*P* = .02).—NCI Monogr 6:357–359, 1988.

Based on cellular kinetic data obtained by monolayer cell culture (*1*), we have shown in L1210 ascitic tumor and in fibrosarcoma in the mouse the results of treatment with x-rays combined with 5-FU and cisplatin (*2,3*). The effectiveness of the different combinations was tested on continuous organotypic culture (*4*). To obtain the most effective growth inhibition, a cisplatin treatment had to be preceded by irradiation, whereas a 5-FU treatment had to be followed by irradiation. Several schedules of radiotherapy have been tested, from 24 hours before to 48 hours after chemotherapy, and several doses of radiation have been studied, from 1.25 Gy to 4 Gy per fraction. Using these data, we have developed a sequential treatment of chemotherapy with low doses of radiation followed by definitive radiotherapy that can be administered to patients with advanced carcinoma (*5*).

MATERIALS AND METHODS

Criteria for entry in the study were: 1) T3–T4 NX M0 or TX N3 M0 primary squamous cell carcinoma of the head and neck region that was surgically unresectable (classified according to the TNM system of the International Union Against Cancer); 2) age <75 years; 3) no prior head and neck irradiation; and 4) creatinine clearance >50 ml/minute. Performance status was graded by the WHO scale, from 0 to 4. Patients were initially examined for the extent of disease by endoscopy, with biopsies, chest x-rays, echography of the liver, and bone scan, as appropriate. Patient characteristics are shown in table 1.

Cisplatin and 5-FU infusions.—Chemotherapy consisted of three cycles of cisplatin and 5-FU. Patients were hospitalized for days 1–7 of each cycle. Each cycle was initiated on a Monday and repeated every 3 weeks. Cisplatin (20 mg/m^2 in 100 ml of 0.9% saline) was infused iv over 20 minutes, followed by a 2-hour infusion of 5-FU (400 mg/m^2), on days 1, 2, 5, and 6. On days 3 and 4, only a 2-hour infusion of 5-FU (400 mg/m^2) was given after irradiation. All patients were treated with iv antiemetics for control of nausea and vomiting.

Radiation parameters.—The low radiation exposures, using cobalt-60, were given on days 3 and 4 before infusion of 5-FU. In most patients, the primary tumor was treated using lateral parallel opposed treatment portals, and only clinically positive nodes were included. Tumor doses were generally calculated in the midline; 3 Gy was given on days 3 and 4 of each cycle, and 18 Gy was given in six fractions over the three cycles. Definitive irradiation was started within 21 days following the last chemotherapy dose of the third cycle; a tumor dose of 60 Gy (midline dose, 2 Gy × 5/wk) was delivered within 6 weeks, using cobalt-60 exclusively or in combination with a 25-MeV photon beam after a total dose of 50 Gy. The target volume encompassed the tumor-bearing structure as well as the bilateral upper and midjugular nodes. The lower portions of the neck received a total dose of 50–60 Gy, and the total dose to the spinal cord never exceeded 45 Gy.

Response criteria.—Measurement was performed clinically and using endoscopy when it was difficult to assess the tumor by clinical examination. A CR was defined as the complete disappearance of all measurable tumor masses for ≥4 weeks. A PR was defined as response less than CR but showing ≥50% reduction in the surface area of all measurable tumor masses for ≥4 weeks. For data analysis, only CR and PR were considered as objective responses.

Toxicity analysis.—All patients were evaluated daily for toxicity during each cycle and at least once per week during the rest period. Only hematologic, ionic, and renal toxic effects were assessed with WHO criteria: grade 0, no toxicity; grade 1, minor toxicity; grade 2, toxicity showing either a bad response to symptomatic treatment or requiring mod-

---

ABBREVIATIONS: 5-FU = 5-fluorouracil; WHO = World Health Organization; CR = complete response; PR = partial response.

---

[1] Department of Radiotherapy (*M. Bolla, J. Borgel,* and *C. Vrousos*) and Department of Otorhinolaryngology (*A. Guenoun* and *C. Junien-Lavillauroy*), Centre Hospitalier Universitaire de Grenoble, France.

[2] Centre Jean Perrin, Clermont-Ferrand, France.

[*] *Reprint requests:* Professor Michel Bolla, Department of Radiotherapy, Centre Hospitalier Universitaire de Grenoble, BP 217 X, 38043 Grenoble Cedex, France.

Table 1.—Patient characteristics[a]

Characteristic	No. of patients (%)
No. treated	36
Performance status	
0	14 (39)
1	13 (36)
2	7 (19)
3	1 (3)
4	1 (3)
Primary site	
Oral cavity	7
Oropharynx	16
Hypopharynx	5
Larynx	6
Nasopharynx	1
Nasal fossa	1
TNM classification	
T stage	
T1	1
T2	2
T3	29
T4	4
N stage	
NX	1
N0	7
N1	10
N2	9
N3	9

[a] All patients were male. Median age was 54 yr (range, 19–74).

ification or delay of treatment; grade 3, major toxicity resulting in discontinuation of treatment; and grade 4, lethal toxicity.

Survival.—The last analysis was performed in September 1986. Survival was calculated from the first day of treatment. Overall survival probability was calculated by computer using the actuarial method of Byar (*6*) with a 95% confidence interval. The chi-square test was required to assess a significant difference between subgroups of patients; it was performed on the exponential adjustment of the actuarial curves.

RESULTS

Thirty-six consecutive patients with a median age of 54 years (range, 19–74) entered this phase II trial. Median follow-up was 11 months (range, 2–30). Twenty-four patients (67%) received three cycles, 8 (22%) received two, and 3 (8%) received four; 1 patient (3%) received one cycle. After chemotherapy with low doses of radiation, the response rate was 47%; 4 patients (11%) had CR and 13 (36%) had PR (table 2). With regard to the 28 patients with clinically positive nodes, the response rate was 46%; 6 (21%) had CR and 7 (25%) had PR. The overall CR rate was 11%; stages of disease in these patients were T3 N0, T3 N2, and T3 N3.

After completion of definitive irradiation, the CR rate reached 30% (11 patients); the PR rate was 30% (11 patients); and the overall response rate was 60% (22 patients). The actuarial local-regional CR rate was 44% (95% confidence interval, 12%–77%) at 1-year follow-up and 27% (0%–60%) at 2 years. The median duration of local-regional CR was 15 months (range, 9–77). Overall survival was 38% (95% confidence interval, 22%–54%) at 1 year and 16% (3%–28%) at 2 years, with a median survival of 10 months (range, 7–14). Two patients remained alive at the time of analysis; 1 patient was clinically free of disease and the other had cancer. One patient was lost to follow-up in CR. Thirty-three patients have died; 32 died of the original disease, and 1 patient died of a second primary tumor. Patients with CR had a 1-year survival of 90% (95% confidence interval, 70%–100%) and a 2-year survival of 38% (5%–72%), with a median survival of 21 months (range, 12–89). Patients with PR or no response had survival rates of 27% and 9%, respectively, and 13% and 7%, respectively, at 1 and 2 years; median survival times are 9 and 6 months, respectively ($P=.6$).

Table 2.—Response after 3 courses of chemotherapy with low doses of radiation

Stage	No. of patients (%) CR	PR	CR+PR	NR
T	4 (11)	13 (36)	17 (47)	19 (53)
N	6 (21)	7 (25)	13 (46)	15 (54)

Toxic effects are shown in table 3. Four patients developed grade 3 hematologic toxicity with leukocytopenia (range, $1\text{–}1.9 \times 10^3/\text{mm}^3$) or thrombocytopenia (range, $25\text{–}49 \times 10^3/\text{mm}^3$). Renal toxicity was transient; only 5.5% of the patients had creatinine levels increasing to values that were 5–10 times the normal values (grade 3). One patient had a decrease of the serum level of sodium to 127 μmol/liter. We are not reporting on upper intestinal complaints because nausea and vomiting were not carefully monitored for all patients. Mucositis was not recorded and graded according to WHO criteria, but clinically, it was the same at the completion of treatment after fractionated irradiation to a total dose of 70 Gy.

DISCUSSION

The radiation used in combination with induction chemotherapy is called "low dose" since the dose of 18 Gy is delivered in six fractions over 9 weeks. Usually, such a protracted dose, in current practice, has no effect on tumors. Nevertheless, the term hypofractionated may be a more accurate term. According to our biologic data, the 3-Gy fractions are required to potentiate both 5-FU and cisplatin, and the cumulative dose must be limited to 78 Gy to avoid late toxic effects to normal tissues. The 6-day regimen of cisplatin plus 5-FU plus low doses of radiation has moderate toxicity but achieves relatively low overall CR and low local control rates, probably because the tumors

Table 3.—Toxic effects

Toxic effect	WHO grade 0	1	2	3	4	Not evaluated
Hematologic	15	11	1	4 (11%)	0	5
Ionic	27	7	1	1 (2.7%)	0	0
Renal	25	3	1	2 (5.5%)	0	5

were not surgically resectable and because 16 of 36 were oropharyngeal tumors. For these locally advanced tumors, this schedule is beneficial only for patients who have an objective response after three cycles; otherwise, the overall response rate after 60-Gy definitive irradiation is not increased. Tumors become radioresistant because both the radiation and the drugs are selective for the same subpopulation of cells, because of the presence of hypoxic cells, and probably because of an increased rate of proliferation. Since January 1985, this therapeutic approach has been used in a randomized trial versus fractionated irradiation with a total dose of 76 Gy given in 38 fractions within 52 days.

REFERENCES

(*1*) DIONET C, COPPEY J: Cytostatic effects of bleomycin and cisplatinum in A 549 human lung tumour cells using autofeeder conditions. Anticancer Res 3:257–262, 1983.

(*2*) DIONET C, VERRELLE P: Curability of mouse L 1210 leukemia combining 5-fluorouracil, *cis*-dichlorodiammine platinum and low doses of X-rays. Cancer Res 44:652–656, 1984.

(*3*) DIONET C, VERRELLE P: Résultats sur la leucémie L 1210 et le fibrosarcome de la souris d'un traitement associant 5 fluorouracile, cis-platinum et faibles doses de rayons X. Presented at the Second Meeting of the European Society of Therapeutic Radiation Oncology. Bordeaux, France, Sept 5–10, 1983.

(*4*) BEAUPIN K, DIONET C: Effects of combined treatment of cisplatin, 5-FU and X-rays on growth of human cancer nodules maintained in continuous organotypic culture. Cancer Res 45:3150–3154, 1985.

(*5*) DIONET C, BOLLA M, DUMESNIL Y, et al: Traitement des tumeurs solides par association séquentielle de 5 fluorouracile, de cisplatinum et de faibles doses de radiothérapie. Bull Cancer (Paris) 70:231, 1983.

(*6*) BYAR D: Analysis of survival data in heterogeneous population. *In* Recent Developments in Statistics. Amsterdam: North Holland, 1977, pp 51–68.

New Strategies for Avoiding Total Laryngectomy in Patients With Head and Neck Cancer[1]

Bhadrasain Vikram,* George J. Bosl, David Pfister, William Assad, Elliot W. Strong, Ronald H. Spiro, Roy B. Sessions, Frank P. Gerold, and Jatin P. Shah[2]

ABSTRACT—Cisplatin-based chemotherapy has yielded high response rates in patients with head and neck cancer but has failed to improve the survival rates in randomized studies. Thus, its place in the management of head and neck cancer remains unclear. We have been investigating whether the combination of chemotherapy and radical irradiation as an alternative to morbid surgery in selected patients might yield an improved quality of life without compromising survival. Between January 1983 and December 1985, we treated 32 patients with epidermoid carcinoma arising from the larynx, base of the tongue, or hypopharynx, using an interdisciplinary regimen, with the objective of avoiding total laryngectomy. All of these patients had large primary tumors that could not have been removed without total laryngectomy. Patients who could be treated by conservation laryngeal surgery were excluded, as were patients who presented with clearly unresectable disease. The regimen employed cisplatin-based chemotherapy together with brachytherapy and external radiation therapy, with highly individualized treatment planning. Seven patients had T2 lesions, 19 had T3, and 6 had T4. The follow-up periods ranged from 8 to 38 months (median, 18). The actuarial survival rate at 2 years is 85%, and the laryngectomy-free rate at 2 years is also 85%. None of the 11 patients with laryngeal cancer has relapsed, and none has required laryngectomy. None of the 12 patients with base of the tongue cancer treated by brachytherapy has relapsed above the clavicles and none has required laryngectomy, but 1 patient has died of complications of treatment. The 4 patients with base of the tongue cancer treated without brachytherapy have relapsed, and 2 have required laryngectomy. All 5 patients with hypopharyngeal cancer remain alive, but 2 have required laryngectomy. The results to date suggest that this treatment approach, which employs chemotherapy and radical irradiation as alternatives to morbid surgery, deserves further exploration. This approach is not recommended, however, for the base of the tongue cancers if brachytherapy is not feasible.—NCI Monogr 6:361–364, 1988.

Either radiation therapy or surgery is effective in the treatment of head and neck cancer in early stages, but in the more advanced stages, the results of treatment have been less satisfactory. In recent years, the combination of surgery and radiation therapy has been gaining acceptance as the treatment most likely to result in consistent local-regional control of advanced but resectable head and neck cancer (*1–4*). Sometimes, however, the price of such treatment is high in terms of morbidity. This is especially true when laryngectomy has to be performed, either due to the location of the primary tumor or due to concerns about postoperative aspiration pneumonia.

For the past 3½ years, we have been exploring a new interdisciplinary strategy with the objective of avoiding total laryngectomy in patients with epidermoid carcinoma of the larynx, hypopharynx, or base of the tongue. It had been apparent to us for some time that external radiation therapy alone, even in doses up to 7,000 rad, was not effective in controlling the disease in the majority of patients when the primary tumor was so advanced that it was not amenable to less than total laryngectomy. The tumor burden in such patients was so extensive that it could not be eradicated without prohibitive toxicity by external radiation therapy. Our new approach, based on radiobiologic principles, utilized brachytherapy and/or chemotherapy prior to external radiation therapy in an effort to decrease the primary tumor burden to a level that might safely be eradicated by external radiation therapy. Enlarged neck nodes were still treated by radical neck dissection in the majority of the patients. In this report, we present our preliminary results.

MATERIALS AND METHODS

Between January 1983 and December 1985, we treated 32 patients with the approach outlined here. Eleven patients had epidermoid carcinoma arising from the larynx, 5 from the hypopharynx, and 16 from the base of the tongue. The T and N stages (*5*) are shown in table 1. Patients with early disease, which technically could be treated by conservation laryngeal surgery, were excluded from this study, as were those who presented with far-advanced disease of questionable resectability. Twenty-one of the patients were men and 11 were women; their ages were 37–72 years (median, 60).

Laryngeal Primary Tumor

Four patients with laryngeal primary tumor had T2 lesions, 6 had T3, and 1 patient had T4. In 8 patients, the primary tumor was supraglottic, and in 3, it was transglottic. All of the patients were treated by cisplatin-based chemotherapy initially. Complete response was defined as complete disappearance of visible tumor at the primary site. Partial response was defined as >50% reduction in the size of the visible tumor. Eight (72%) of the patients had

---

[1] Supported by Public Health Service grant CA-05826 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and the Enid Ancell Clinical Studies Fund.

[2] Department of Radiation Oncology (*B. Vikram* and *W. Assad*), Solid Tumor Service, Department of Medicine (*G. J. Bosl* and *D. Pfister*), and Head and Neck Service, Department of Surgery (*E. W. Strong, R. H. Spiro, R. B. Sessions, F. P. Gerold,* and *J. P. Shah*), Memorial Sloan-Kettering Cancer Center, New York, NY.

* *Reprint requests:* Bhadrasain Vikram, M.D., Department of Radiation Oncology, Beth Israel Medical Center, First Ave. and 16th St., New York, NY 10003.

TABLE 1.—T and N stages of 32 patients with head and neck cancer[a]

Nodal stage	Stage of primary tumor T1	T2	T3	T4	Total
N0	0	1	10	1	12 (38%)
N1	0	2	3	1	6 (19%)
N2	0	2	1	0	3 (9%)
N3	0	2	5	4	11 (34%)
Total	0	7 (22%)	19 (60%)	6 (18%)	32 (100%)

[a] Sixteen patients (50%) had stage III disease, and 16 had stage IV disease. Staging was performed according to the criteria of the American Joint Committee on Cancer, 1980. Values = No. of patients.

complete response at the primary site, and 3 (28%) had partial response. All of these patients were then treated by external radiation therapy delivering a total dose of 7,000 rad in 7 weeks to the primary site. Both sides of the neck were treated electively in all patients to 5,000 rad in 5 weeks, with a boost to areas of palpable nodal disease. None of these patients had radical neck dissection.

Hypopharyngeal Primary Tumor

One patient with hypopharyngeal primary tumor had a T2 lesion, 2 patients had T3 lesions, and 2 had T4. All of these patients were treated with chemotherapy initially. One had complete response and 2, partial response; 2 failed to respond. The patient with complete response was treated at the primary site with external radiation therapy to a total dose of 7,000 rad in 7 weeks. The 2 patients with partial response were treated with brachytherapy to 3,000 rad in 3 days, followed by external radiation therapy to 5,000 rad in 5 weeks; the total dose to the primary site was 8,000 rad. The 2 patients who did not respond to chemotherapy were treated by total laryngectomy followed by postoperative external radiation therapy to 6,000 rad in 6 weeks. Four of the 5 patients also underwent unilateral radical neck dissection.

Base of the Tongue Primary Tumor

Two patients with base of the tongue primary tumor had T2 lesions, 11 had T3, and 3 had T4. Ten of the 16 patients were treated with chemotherapy initially. Five of them (50%) had complete response and 2 (20%), partial response; 3 (30%) failed to respond. Twelve patients were treated by brachytherapy (6 after chemotherapy and 6 without any chemotherapy) to a dose of 3,000 rad in 3 days, followed by external radiation therapy to a dose of 5,000 rad in 5 weeks; the total dose to the primary site was 8,000 rad. Four patients were not treated with brachytherapy. After chemotherapy, 3 of these patients were treated by external radiation to 7,000 rad in 7 weeks, and 1 had resection of the primary tumor without laryngectomy and then received postoperative external radiation therapy. All of the patients received elective external radiation therapy to both sides of the neck. In addition, 5 patients had unilateral radical neck dissection, and 1 patient had bilateral radical neck dissection.

Chemotherapy

Two different regimens of chemotherapy were utilized during the course of this study. Regimen A was given to the first 12 patients (*6*). It consisted of cisplatin (120 mg/m^2) on day 1, with hydration and mannitol-induced diuresis, and vinblastine (4 mg/m^2) on days 1, 8, 15, and 22. Regimen B was administered to the subsequent 16 patients and consisted of cisplatin and bleomycin. Cisplatin in 3% saline (37.5 mg/m^2 per day) was given on days 1–5, and bleomycin (10 mg/m^2) was given by iv push on day 1, followed by bleomycin (10 mg/m^2) given by continuous iv infusion on days 1–5. If, at any time, there was evidence of disease progression or of excessive toxicity, chemotherapy was discontinued and the patient proceeded with appropriate surgical treatment. Otherwise, chemotherapy was continued for three cycles. Six patients, all with base of the tongue carcinoma, who were seen during the early part of the study, received no chemotherapy but were treated with brachytherapy and external radiation therapy with or without neck dissection, as appropriate. One patient had a T2 primary tumor, 3 had T3 primary tumors, and 2 had T4.

Brachytherapy

Our technique for interstitial implantation of the base of the tongue has been previously published (*7,8*). The technique for interstitial implantation of tumors in the hypopharynx involves the placement of hollow stainless-steel needles through the midline of the neck at intervals of 1–1.5 cm, in a posterior direction. The most superior needle is introduced just superior to the level of the hyoid bone, and additional needles are introduced through the thyroid notch, through the cricothyroid membrane, and between the tracheal rings. The needles extend posteriorly into the prevertebral fascia. Polyethylene afterloading catheters are introduced through the needles, and the needles are removed. Additional planes are created lateral to this midline plane on one or both sides of the midline, as necessary. The second plane is medial to the major vessels and extends posteriorly to the level of the vertebral bodies. The third plane is lateral to the major vessels and extends posteriorly as far back as the transverse processes. If necessary, additional sagittal planes are created in the lateral part of the neck, parallel to the previous planes. Finally, a coronal plane is created at approximately 90° to these previous planes, in order to "cross" the distal end of the implant. This is done by introducing hollow needles through the lateral part of the neck posterior to the major vessels in a medial direction, so that the needles traverse the prevertebral fascia. Again, afterloading catheters are introduced, and the hollow needles are removed.

External Radiation Therapy

Our technique for external radiation therapy consists of a three-field arrangement. The primary tumor and the upper part of the neck are irradiated through parallel opposing lateral, shaped portals, utilizing a cobalt-60 beam. The lower part of the neck, the supraclavicular areas, and the superior mediastinum, if indicated, are treated through a single anterior portal. Appropriate technical measures are adopted to prevent overdosage to the spinal cord while avoiding underdosage to the regional lymphatics. In patients who receive a boost to the primary tumor by external beam, the portals are reduced to encompass the primary tumor after 5,000 rad have been delivered to the neck. In patients who require an external therapy boost to enlarged cervical lymph nodes and in whom these lymph nodes are not included in the boost volume of the primary tumor, the additional dose to the lymph nodes is delivered by means of electron beams.

RESULTS

The median follow-up period is 20 months (range, 10–40). Thirty of the 32 patients (94%) are alive, and in 28 (87.5%), the larynx has been preserved (fig. 1).

None of the 11 patients with laryngeal cancer has relapsed, and none has required laryngectomy. All are alive and free of disease (median follow-up, 16 mo; range, 10–28). None of the 5 patients with hypopharyngeal cancer has relapsed, and all are alive free of disease (median follow-up, 13 mo; range, 10–25); however, as mentioned previously, 2 of the 5 had total laryngectomy when they failed to respond to chemotherapy.

Of the 16 patients with base of the tongue cancer, 12 were treated with brachytherapy. None of the 12 patients has relapsed above the clavicles (median follow-up, 26 mo; range, 10–40), and none has required laryngectomy, but 1 patient died of complications of treatment, and another died of distant metastases. In contrast, the 4 patients with base of the tongue cancer who were treated without brachytherapy have relapsed above the clavicles; 2 required total laryngectomy.

One patient has died of complications related to the treatment. The patient developed severe peripheral neuropathy during chemotherapy and also had persistent aspiration during local therapy. He succumbed to aspiration pneumonia 6 months after the start of treatment, although he was apparently free of disease.

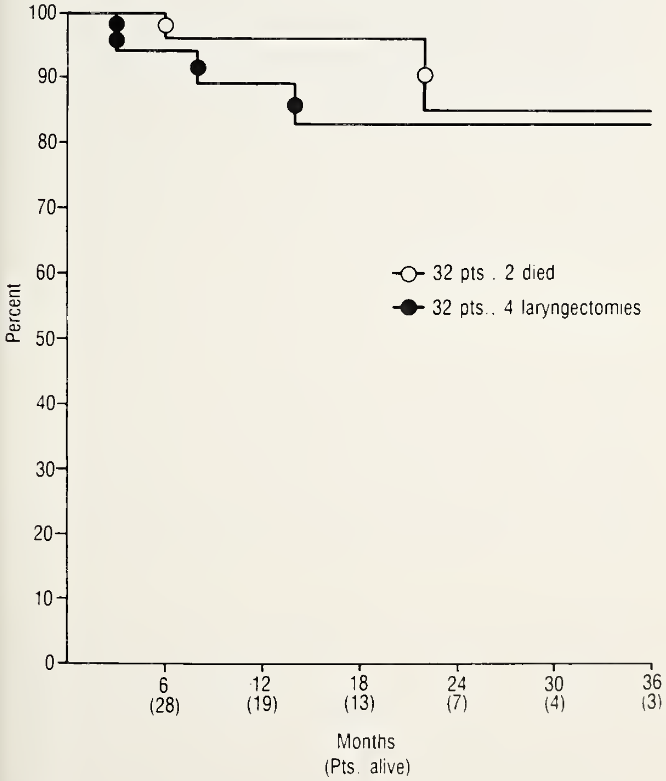


FIGURE 1.—Proportion of patients alive (*open circles*) and proportion of patients without laryngectomy (*closed circles*); Kaplan-Meier method. No. in parentheses indicates No. of patients at risk.

TABLE 2.—Best response of primary tumor in 26 patients who received chemotherapy[a]

Response	Stage of primary tumor			Total
	T2	T3	T4	
Complete	6 (100%)	7 (44%)	1 (25%)	14 (54%)
Partial	0 (0%)	6 (37.5%)	1 (25%)	7 (27%)
None	0 (0%)	3 (19%)	2 (50%)	5 (19%)
Total	6	16	4	26

[a] Values = No. of patients.

The usual toxic effects of cisplatin-based chemotherapy and radiation therapy were also seen during this study. With regimen A (cisplatin and vinblastine), the toxicity was mainly nausea, vomiting, and myelosuppression, which were rarely severe (*6*). There were no drug-related deaths.

Regimen B (cisplatin and bleomycin) was associated with mild myelosuppression, mild ototoxicity, and little nephrotoxicity, but there was occasional major neuropathy, which was probably associated with the fatality described. Details of this regimen will be published elsewhere (*9*).

DISCUSSION

The treatment strategy described here required highly individualized and coordinated interdisciplinary treatment planning. The results obtained to date (fig. 1) suggest, however, that it might be well worth the trouble. Whereas in the past, virtually all of these patients would have undergone total laryngectomy, in this study, the larynx has been preserved in 28 of the 32 patients. This is most encouraging and suggests that preservation of the larynx by such multimodality therapy deserves further exploration. As additional experience is gained, it will be necessary to fine tune the treatment to maximize the therapeutic ratio. Some of the issues that are already emerging are discussed here.

Response to Chemotherapy

Table 2 shows the response to chemotherapy. It is notable that the maximum response to chemotherapy was apparent shortly after the second course and that few additional responses or complete responses were achieved after administration of the third course. By the time local therapy was instituted, there was a slight decrease in the proportion of responders and complete responders. These data raise doubts about the value of the third course of chemotherapy. A decrease in the number of courses of chemotherapy might also have a favorable impact on the toxicity of chemotherapy. At the present time, our data do not show a correlation between response to chemotherapy and subsequent prognosis. The role of chemotherapy remains unclear among the patients with base of the tongue cancer who underwent brachytherapy, since neither the 6 patients who underwent brachytherapy without chemotherapy nor the 6 whose brachytherapy was preceded by chemotherapy have had local recurrence. We found, however, that a good response to chemotherapy in bulky primary tumors facilitated technically satisfactory implants, as others have reported (*10*).

Role of Brachytherapy

None of the 12 patients with base of the tongue cancer treated with brachytherapy has relapsed above the clavicles, while the 4 treated without brachytherapy have relapsed. The decision not to use brachytherapy in 3 of these 4 patients was based on personal preference rather than any scientific basis. In 1 patient, the decision was made not to use brachytherapy because of multifocal appearance of the tumor. It now appears that this treatment approach is not likely to succeed in patients with base of the tongue cancer if brachytherapy is not employed, even though there may have been complete response to chemotherapy, as was the case in 2 of these 4 patients. We think that the favorable results seen with brachytherapy are related to the approximately 1,000-rad additional dose of radiation received by the primary tumor, bringing the total dose to 8,000 rad. This is in keeping with the well-known dose-response relationships in epidermoid carcinomas of the upper aerodigestive tract.

We have not used brachytherapy in patients with laryngeal primary tumor, and at the present time, we do not see the need to do so, in view of the fact that none of these patients has relapsed after chemotherapy and external radiation therapy. It is worth noting that the tumor burden with a T3 laryngeal primary tumor is generally smaller than with a T3 base of the tongue primary tumor. Therefore, the cytoreduction produced by chemotherapy preceding 7,000 rad of external radiation therapy might be adequate in laryngeal primary tumor but not in base of the tongue primary tumor.

As part of this study, we have also made an effort to develop brachytherapy techniques applicable to hypopharyngeal primary tumor. It is interesting that the 2 patients with hypopharyngeal primary tumor who had only partial response to chemotherapy and were then treated by brachytherapy followed by external irradiation remain free of relapse so far. However, many additional patients and longer follow-up will be necessary to evaluate the safety and effectiveness of brachytherapy in the hypopharynx.

REFERENCES

(*1*) Vikram B, Strong EW, Shah JP, et al: Elective postoperative radiation therapy in stages III and IV epidermoid carcinoma of the head and neck. Am J Surg 140:580–584, 1980.

(*2*) Vikram B, Strong EW, Shah JP, et al: Failure at the primary site following multimodality treatment in advanced head and neck cancer. Head Neck Surg 6:720–723, 1984.

(*3*) Fletcher GH: Combination of irradiation and surgery. *In* Textbook of Radiotherapy (Fletcher GH, ed). Philadelphia: Lea & Febiger, 1980, pp 219–224.

(*4*) Cachin Y, Eschwege F: Combination of radiotherapy and surgery in the treatment of head and neck cancer. Cancer Treat Rev 2:177–191, 1975.

(*5*) American Joint Committee on Cancer: Staging of Cancer of Head and Neck Sites and of Melanoma. Chicago: American Joint Committee on Cancer, 1980.

(*6*) Leitner SP, Bosl GJ, Strong EW, et al: A pilot study of cisplatin, vinblastine as the initial treatment of advanced head and neck cancer. Cancer 58:1014–1017, 1986.

(*7*) Vikram B, Hilaris BS: A non-looping afterloading technique for interstitial implants of the base of the tongue. Int J Radiat Oncol Biol Phys 7:410–422, 1981.

(*8*) Vikram B, Strong EW, Shah JP, et al: A non-looping afterloading technique for base of tongue implants—Results in the first 20 patients. Int J Radiat Oncol Biol Phys 11:1853–1855, 1985.

(*9*) Pfister D, Bosl GJ, Tauer K, et al: Neoadjuvant chemotherapy of advanced head and neck cancer with high dose cisplatin and bleomycin. Proc ASCO 5:140, 1986.

(*10*) Arcangeli G, Nervi C, Righini R, et al: Combined radiation and drugs: The effect of intra-arterial chemotherapy followed by radiotherapy in head and neck cancer. Radiother Oncol 1:101–107, 1983.

Clinical Experience With High-dose Cisplatin and Radiation for Treatment of Epithelial Malignancies[1]

Christopher T. Coughlin[2,3,*]

ABSTRACT—Over the past 5 years, 129 patients have been treated with a combination of high-dose cisplatin (CDDP) and radiation for locally advanced epithelial malignancies. The CDDP was administered at a dose of 100 mg/m^2 by iv infusion over one-half hour, no more than 1 hour before irradiation, every 3 weeks during a full course of external beam irradiation. An attempt was made to take advantage of the interaction of high-dose CDDP and radiation. Tumor systems studied included head and neck, ovary, lung, cervix, and prostate. Median survival times are as follows: squamous cell carcinoma of the head and neck (trial 1), 36 months; ovarian carcinoma, 19; and squamous cell carcinoma of the lung, 14. Median survival has not yet been reached in trials of squamous cell carcinoma of the head and neck (trial 2), cervical carcinoma, or adenocarcinoma of the prostate.—NCI Monogr 6:365–367, 1988.

Cisplatin has been shown to be a radiation potentiator in a variety of in vitro systems (*1–12*). The work of Douple and Richmond (*3–5*) in the radiobiology laboratories of the Dartmouth-Hitchcock Medical Center has led to the development of clinical trials for locally advanced epithelial malignancies in an attempt to take advantage of the dual role of cisplatin as an active chemotherapeutic agent as well as a radiation potentiator. The initial clinical trials began in early 1981 with Dartmouth Medical School protocols for the treatment of advanced squamous cell carcinoma of the head and neck, locally advanced squamous cell carcinoma of the lung, ovarian carcinoma, cervical carcinoma, and adenocarcinoma of the prostate. In general, these protocols have involved administration of high-dose cisplatin by iv infusion every 3 weeks within a standard 5- to 7-week course of external beam radiation therapy; the infusions were given within 1 hour prior to irradiation.

MATERIALS AND METHODS

Six protocols were tested (table 1). The first protocol was designed to treat stage III and IV squamous cell carcinoma of the head and neck, using both chemotherapy and a standard course of radiation therapy. The initial cycle of chemotherapy consisted of one half-hour cisplatin infusion, followed by daily bleomycin infusions over the next 4 days. However, bleomycin was eliminated from the protocol because it confused evaluation of efficacy and did not clearly show any additional activity over cisplatin alone. Therefore, subsequent cycles consisted of cisplatin only. Patients had simulation on or about day 15, so treatment planning was completed by the time of the second large dose of cisplatin on day 21, which was administered within 1 hour before the first dose of radiation. For the next 5 weeks, weekly doses of cisplatin were given before the first of the daily radiation treatments for that week. At the close of this period, patients were again evaluated. Those who had been deemed inoperable initially or who showed a major tumor reduction underwent a final 2 weeks of external beam radiation therapy without further cisplatin. Those who had been deemed operable initially or who showed only minimal tumor response underwent surgical resection.

A second-generation head and neck protocol for advanced local malignancies was developed utilizing an entirely different treatment sequence to evaluate the toxicity of the combined modalities used solely in an adjuvant setting. In this protocol, patients received cisplatin on day 1, followed on day 21 by complete surgical resection of the primary tumor and adjacent clinically involved lymph nodes. At 3–6 weeks postoperatively, patients received a standard 5-week course of radiation, with two additional infusions of cisplatin given 3 weeks apart.

A third protocol was designed to evaluate the toxicity of cisplatin and radiation in surgical stage III ovarian adenocarcinoma. Patients had initial exploratory and debulking surgery resulting in <2 cm of residual disease if possible. They then received four cycles of cisplatin at 3- to 4-week intervals, followed by second-look laparotomy for assessment of response and further debulking if necessary. During the next 3 weeks, 2,100 cGy was delivered to the entire abdomen and pelvis, supplemented by 3,000 cGy to the pelvis alone over a final 3 weeks. Two additional doses of cisplatin were given, one at the start of the "belly bath" irradiation and the other at the start of pelvic irradiation.

A fourth protocol was devised to treat patients with locally advanced squamous cell carcinoma of the lung confined to the chest, a subset that does not fit readily into most other protocols but includes a significant portion of patients who may die from local disease only. Over a 6-week period, these patients were given two large doses of cisplatin as well as radiation to both sides of the neck and mediastinum down to the level of the diaphragm.

A fifth protocol was designed for bulky, local squamous cell carcinoma of the cervix. Over a 5-week period, patients received radiation as well as two large doses of cisplatin; the doses of cisplatin were administered within 1 hour prior to irradiation. This sequence was followed by two tandem and colpostat insertions at 2-week intervals.

The final protocol was designed to treat patients with high-grade, poor-risk, locally advanced adenocarcinoma of

---

[1] Supported by Public Health Service grant CA-23108 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Department of Radiation Therapy, Dartmouth-Hitchcock Medical Center, Hanover, NH.

[3] I thank Jo Strohbehn for assistance in data collection and Bradley J. Danin for assistance in biostatistical analysis.

* *Reprint requests:* Christopher T. Coughlin, M.D., Department of Radiation Therapy, Dartmouth-Hitchcock Medical Center, 2 Maynard St., Hanover, NH 03756.

TABLE 1.—Results of simultaneous treatment with high-dose cisplatin and high-dose external beam radiation for advanced epithelial malignancies

Disease site	Disease stage	No. of patients	Dose and schedule: Cisplatin	Dose and schedule: Radiotherapy	Median survival[a] (mo)
Head and neck (trial 1)	III–IV	54	100 mg/m^2 every 3 wk×2, then 20 mg/m^2 every wk×5	200 cGy/day×25, then surgery or 200 cGy/day×10	36
Head and neck (trial 2)	III–IV	17	100 mg/m^2, surgery, then 100 mg/m^2 every 3 wk×2	200 cGy/day×25	NR
Ovary	III	16	100 mg/m^2 every 3 wk×4, 2nd-look laparotomy, then 100 mg/m^2×1	150 cGy/day×14, then boost to pelvis of 200 cGy/day×15	19
Lung	III	20	100 mg/m^2 every 3 wk×3	200 cGy/day×30	14
Cervix	IIB/IIIB	16	100 mg/m^2 every 3 wk×3	200 cGy/day×25+2 tandem and colpostat insertions	NR
Prostate	D	6	100 mg/m^2 every 3 wk×3	200 cGy/day×35	NR

[a] NR=not reached.

the prostate with disease limited to pelvic lymph nodes at staging laparotomy. During a period of approximately 7 weeks, these patients received radiation to the prostate, supplemented by three large doses of cisplatin given at 3-week intervals.

RESULTS

Of the 54 evaluable patients entered in the first head and neck study, 28 died, with a minimum follow-up of 30 months. Of these 54 patients, 5 died with local failure only, 5 died with local and distant failure, 6 died with distant failure only, and 9 died of unrelated causes (seizure disorder, 1 patient; myocardial infarction, 2 patients; postoperative wound-healing problems, 1; suicide, 2; pneumonia, 1; disseminated intravascular coagulation, 1; and metastatic breast cancer, 1). Toxicity for the initial 25 patients entered in this study has been previously reported (*13*). An analysis of the group that had surgery for consolidation therapy has also been completed, along with pathological evaluation of the surgical specimens (*14*).

Of the 17 patients entered in the second-generation head and neck protocol for advanced local malignancies, 13 are alive without evidence of disease. There have been 4 deaths; 1 patient died of liver and lung metastases with good local control 14 months after the initiation of treatment, and the other 3 patients died as a result of surgery within 3 months after entering the study. These patients were treated with only a single dose of cisplatin and surgery.

There were 16 patients entered in the protocol for stage III ovarian carcinoma. Eleven died within 5–21 months after treatment; 5 are still alive 28–36 months after treatment. Morbidity in this protocol has been unacceptably high; 7 patients required subsequent surgical intervention for small bowel obstruction. Ten of these 16 patients also had some degree of cisplatin-induced peripheral neuropathy, although in most cases, this was minor. Neuropathy may have been dose related, since patients in this protocol received higher doses of cisplatin than those in the other protocols. The protocol has been discontinued due to toxicity, and its efficacy has yet to be proven.

Twenty patients were entered in the protocol for locally advanced squamous cell carcinoma of the lung. Five remain alive 28–55 months after treatment. Fifteen patients died 1–21 months after treatment, two-thirds with locoregional failure and one-third with metastatic disease only. These results are encouraging because although these patients had either bulky disease at presentation or supraclavicular adenopathy, the toxicity was quite acceptable. Esophagitis appeared to be the only significant toxic effect during this course of treatment (8 patients), with no increased incidence of pulmonary, cardiac, or neurological complications. In this protocol, care was taken to treat the mediastinum to a total dose of 4,500 cGy and to spare the spinal cord above that dose.

Sixteen patients were entered in the protocol for locally advanced squamous cell carcinoma of the cervix. Six died with disease 5–21 months after treatment, 3 with metastatic disease and 3 with locoregional recurrence. Ten patients are still alive 24–42 months after treatment, and there was no evidence of mucositis, bowel or urinary tract problems, or skin changes within the radiation portal. Median survival for this study has yet to be reached.

Finally, only 6 patients were entered in the protocol for treatment of locally advanced, high-grade, poor-risk adenocarcinoma of the prostate. Two patients died of metastatic disease primarily in the bone at 7 and 31 months after treatment, although local control was achieved. Four patients are alive without any evidence of disease 44–50 months after treatment. Typically, these patients would be expected to have some type of recurrence within 18 months.

DISCUSSION

The major objective of these studies was to combine high-dose cisplatin simultaneously with standard high-dose external beam radiation for the treatment of advanced epithelial malignancies (table 1). The studies were limited to tumors that manifest locally advanced disease, since the intent was to improve local control with this combined modality approach. The radiation fractionation schema were standard, so the only new variable was the addition of cisplatin. Apart from ovarian carcinoma, the toxic effects for each cancer studied were found to be entirely acceptable. Administration of cisplatin did not result in any damage beyond that predicted on the basis of drug metabolism and local toxic effects from radiation therapy.

The initial clinical trial of advanced head and neck cancer utilized weekly small doses of cisplatin in addition to the larger doses. Based on the responses of the initial 25 patients, it appears that these small doses added only to the toxicity of this regimen. The next 27 patients were treated with high-dose cisplatin alone, which was given every 3 weeks during the irradiation sequence, resulting in equal efficacy and much less toxicity. Predictably, all patients in both head and neck studies had mucositis, nausea and vomiting, and weight loss. With more aggressive nutritional support and better antiemetic regimens, these symptoms became more tolerable as the initial trial progressed.

Our experience in the initial head and neck trial raised a question as to the ability of wounds to heal after 4,800-cGy external beam radiation and cisplatin. This triggered a subsequent animal study, which showed no effect on wound healing with this combination of agents, in terms of epithelial closure, desquamation, and infectious wound complications (*15*). The major toxic effect was the suppression of adequate nutritional intake. In the second head and neck study, there was no adverse effect on wound healing, and patients were able to tolerate a full postoperative course of adjuvant radiation therapy and cisplatin.

The trial of locally advanced squamous cell carcinoma of the lung had no unforeseeable toxic effects (*16*). We saw no radiation pneumonitis, although this could have been predicted to some degree because most patients had very large tumor masses at the time of presentation, necessitating generous radiation fields to encompass all known disease. Esophagitis occurred at the time it would have been expected as a result of external beam radiation alone. There were no adverse effects on skin, spinal cord, heart, or bronchial mucosa.

The patients with squamous cell carcinoma of the cervix had no toxic effects from the combination treatment. There was no unforeseen diarrhea, dysuria, or premature reddening of the skin within the radiation portal.

Most patients with ovarian carcinoma experienced significant toxic effects from the combination of extensive surgical debulking and belly bath irradiation. Aside from small bowel problems, however, these toxic effects were no greater than would have been expected from this type of surgery and large-field irradiation, and we would not ascribe any of the toxic effects experienced to the addition of cisplatin.

Patients with adenocarcinoma of the prostate also showed no toxic effects from the treatment combination. Although this was a very small trial, all patients with high-grade D1 disease would be expected to have recurrence within 18 months; yet only 2 of the 6 patients developed recurrence, and both of these exhibited good local control at the time they developed bone metastases. The patient who had 7-month survival after irradiation probably had metastatic disease in the bone when treatment was initiated, which at the time was believed to be Paget's disease of the bone. The other patient had good local control for a substantial period of time (10 mo) prior to developing fatal bony metastases.

The concept of radiation potentiation may be very difficult to prove clinically and will require well-constructed trials with substantial numbers of patients in each trial. We are encouraged by the general lack of toxic effects in the phase I trials and have continued to pursue interactions between single-agent drugs and radiation in the tumor sites tested. Larger doses of platinum-containing compounds are now feasible due to the availability of second-generation platinum analogs. Single-drug/radiation interactions will be very useful in the future, eventually leading to the logical construction of combination chemotherapy regimens.

REFERENCES

(*1*) Richmond RC, Powers EL: Radiation sensitization of bacterial spores by *cis*-dichlorodiammine platinum (II). Radiat Res 68:251–257, 1976.

(*2*) Richmond RC, Simic MG: Effect of radiation on *cis*-dichlorodiammine platinum (II) and DNA in aqueous solution. Br J Cancer 37(suppl III):20–23, 1978.

(*3*) Douple EB, Richmond RC: Platinum complexes as radiosensitizers of hypoxic mammalian cells. Br J Cancer 37:98–102, 1978.

(*4*) Douple EB, Richmond RC: A review of platinum complex biochemistry suggests a rationale for combined platinum-radiotherapy. Int J Radiat Oncol Biol Phys 5:1335–1339, 1979.

(*5*) Douple EB, Richmond RC: Radiosensitization of hypoxic tumor cells by *cis*- and *trans*-dichlorodiammine platinum (II). Int J Radiat Oncol Biol Phys 5:1369–1372, 1979.

(*6*) Carde P, Laval F: Effect of *cis*-dichlorodiammine platinum (II) and X-rays on mammalian cell survival. Int J Radiat Oncol Biol Phys 7:929–933, 1981.

(*7*) Dritschilo A, Piro AJ, Kelman AD: The effect of *cis*-platinum on the repair of radiation damage in plateau phase Chinese hamster (V-79) cells. Int J Radiat Oncol Biol Phys 5:1345–1349, 1979.

(*8*) Richmond RC: Toxic variability and radiation sensitization by dichlorodiammineplatinum(II) complexes in *Salmonella typhimurium* cells. Radiat Res 99:596–608, 1984.

(*9*) Richmond RC, Khokhar AF, Teicher BA, et al: Toxic variability and radiation sensitization by Pt (II) analogs in *S. typhimurium* cells. Radiat Res 99:609–626, 1984.

(*10*) Richmond RD, Zimbrick JD, Hykes DL: Radiation-induced DNA damage and lethality in *E. coli* as modified by the antitumor agent *cis*-dichlorodiammine platinum (II). Radiat Res 71:447–460, 1977.

(*11*) Szumiel I, Nias AHW: The effect of combined treatment with a platinum complex and ionizing radiation on Chinese hamster ovary cells in vitro. Br J Cancer 33:450–458, 1976.

(*12*) Zimbrick JD, Sukrochana A, Richmond RC: Studies on radiosensitization of *E. coli* cells by *cis*-platinum complexes. Int J Radiat Oncol Biol Phys 5:1351–1354, 1979.

(*13*) Coughlin CT, Grace M, O'Donnell JF, et al: Combined modality approach in the management of locally advanced head and neck cancer. Cancer Treat Rep 68:591–597, 1984.

(*14*) Coughlin CT, Richmond RC: Platinum based combined modality approach for locally advanced head and neck carcinoma. Int J Radiat Oncol Biol Phys 11:915–919, 1985.

(*15*) Morain WD, Richmond RC, Jacobs NJ, et al: Preoperative radiation potentiation with cisplatin: Effect on rate of wound infection. Am J Surg. In press.

(*16*) Coughlin CT, Delprete SA, Grace MP, et al: Cisplatin and radiation therapy for locally advanced squamous cell carcinoma of the lung. Cancer Treat Rep 70:643–645, 1986.

Tolerance and Preliminary Results of Simultaneous Therapy With Radiation and Cisplatin for Advanced Cervical Cancer

D. J. Monyak,[1,*] L. B. Twiggs,[2] R. A. Potish,[1] L. L. Adcock,[2] J. E. Savage,[2] G. O. Downey,[2] L. F. Carson,[2] and K. A. Prem[2]

ABSTRACT—From 1982 through 1985, 49 women with advanced cervical cancer were treated with concurrent radiation and cisplatin to assess tolerance to the combined modality regimen and its efficacy in comparison to historical controls at the same institution. Eligibility criteria included clinical stage IV disease; para-aortic lymph node metastases; and bilateral, unresectable, and/or multiple (>3) pelvic node metastases. Nodal status was assessed by extraperitoneal surgical staging in the majority of patients with clinical stage I-IVA disease. There was no increase in acute or chronic toxicity compared to historical controls. No patient required reduction of the planned dose of radiation because of acute toxicity. Comparison with historical controls suggests a modest improvement in relapse-free survival in the cisplatin-treated group with positive para-aortic nodes (59% vs. 54% at 2 yr and 59% vs. 42% at 3 yr). We conclude that adjuvant cisplatin can be given simultaneously with radiation for advanced cervical cancer without excessive toxicity or compromise of the radiation regimen. However, confirmation of the modest improvement in relapse-free survival rates for patients with positive para-aortic nodes and identification of other subsets of patients who may benefit will require a prospective randomized trial.—NCI Monogr 6:369-373, 1988.

Results with the standard treatment of radiation alone in patients with advanced cancer of the uterine cervix have been unsatisfactory because of the development of distant metastases beyond the radiation portals and the failure of radiation to control the known local-regional disease (*1-3*). The use of adjuvant cisplatin with radiation therapy is attractive because of 1) the ability of cisplatin to cause regression of cervical cancer when used as a single agent (*4-6*), thus allowing potential eradication of distant foci of disease, and 2) its ability to sensitize tumors to radiation (*7-17*), thus allowing potential improvement in local-regional control. In addition, cisplatin has relatively little bone marrow toxicity (*18*).

In 1982, the University of Minnesota began a trial of adjuvant cisplatin given concurrently with radiation to evaluate the toxicity and the efficacy of this approach. Preliminary results on smaller numbers of patients have been previously reported (*19,20*).

MATERIALS AND METHODS

Protocol Eligibility

Patients were eligible for entrance in the study if they met one or more of the following tumor criteria:

1) stage IV disease;
2) para-aortic lymph node metastases;
3) unresectable pelvic lymph node metastases;
4) involvement of multiple (>3) pelvic lymph nodes; and
5) bilateral involvement of pelvic lymph nodes.

Laboratory criteria required were:

1) WBC count >4,000/mm^3;
2) platelet count >90,000/mm^3; and
3) serum creatinine <1.5 mg/dl.

A total of 49 patients entered the study from 1982 to the end of 1985. No patient had received any prior treatment. All patients had measurable disease.

Other Patient Characteristics

Of the 49 patients, 3 (6%) were 20-30 years of age; 11 (22%) were 30-40; 10 (20%) were 40-50; 13 (27%) were 50-60; 9 (18%) were 60-70; and 3 (6%) were 70-80. Eleven (22%) of the patients had clinical stage IB disease; 13 (27%), stage IIB; 11 (22%), stage III; 3 (6%), stage IVA; and 11 (22%), stage IVB.

Histological examination showed that 29 (59%) of the patients had squamous cell carcinoma; 5 (10%), adenocarcinoma; 10 (20%), adenosquamous carcinoma; 4 (8%), undifferentiated carcinoma; and 1 patient (2%), sarcoma. Of the 48 carcinomas, 46 were graded: 1 (2%) was grade 1/3; 18 (39%) were grade 2/3; and 27 (59%) were grade 3/3. The grading system used was that of the International Federation of Gynecology and Obstetrics.

In 33 (87%) of 38 patients with clinical stage I-IVA disease, nodal status and the presence of peritoneal seeding were evaluated through extraperitoneal surgical staging. Three patients with stage I-IVA disease had nodal involvement documented by lymphangiogram only. Two patients with bulky stage IIIB disease were entered in the study without surgical or radiological evaluation of nodal status; they are excluded from analysis of absolute survival and relapse-free survival by nodal status. The distribution of pathological nodal status by clinical stage is given in table 1.

Radiation Therapy

Patients with stage IB-IVA disease received 8,000-8,500 cGy to point A (2 cm superior and 2 cm lateral to the cervix) and 6,000 cGy to point B (2 cm superior and 5 cm

---

[1] Department of Therapeutic Radiology-Radiation Oncology, Health Science Center, University of Minnesota Hospital, Minneapolis.

[2] Division of Gynecologic Oncology, Department of Gynecology and Obstetrics, University of Minnesota Medical School, Minneapolis.

* *Reprint requests:* D. J. Monyak, M.D., Department of Therapeutic Radiology-Radiation Oncology, Box 494, Health Science Center, University of Minnesota Hospital, Minneapolis, MN 55455.

TABLE 1.—Pathological lymph node status and presence or absence of peritoneal seeding vs. clinical stage[a]

Nodal involvement	Stage				Total
	IB	IIB	III	IVA	
Pelvic only	7	2	2	2	13
Para-aortic ± pelvic	3	7	5	—	15
Peritoneal seeding only	—	1	—	—	1
Para-aortic + pelvic + peritoneal seeding	1	2	1	1	5
Total	11	12	8	3	34[b]

[a] Values = No. of patients.

[b] Includes all 33 patients who underwent extraperitoneal staging laparotomy and 1 patient who had positive needle biopsy of clinically positive node on lymphangiogram.

lateral to the cervix), using a combination of pelvic external beam therapy and two intracavitary Fletcher cesium-137 radioisotope applications. Radiation at a dose of 4,550–4,900 cGy was given to the low para-aortic nodes (to the level of the second lumbar vertebra) if pelvic nodal disease was present and to the high para-aortic nodes (minimum coverage to the level of the 10th thoracic vertebral body) if para-aortic nodal disease was present. If peritoneal spread was documented through positive peritoneal fluid cytology or peritoneal implants, 1,500–2,000 cGy was given to the whole abdomen. In giving pelvic and para-aortic external beam radiation, daily fractions of 175 cGy were used, and in giving abdominal external beam radiation, daily fractions of 100 cGy were used.

In 7 (14%) of the patients, the whole pelvis only was treated; in 14 (29%), the whole pelvis and low para-aortic nodes were treated; in 21 (43%), the whole pelvis and entire para-aortic chain; in 1 patient, the whole pelvis and whole abdomen; and in 5 (10%), the whole pelvis, the entire para-aortic chain, and the whole abdomen.

The planned dose of radiation was completed by 48 patients. One patient stopped treatment after six fractions of radiation and one dose of cisplatin, against medical advice. This patient had a stage IIIA squamous cell carcinoma with involved pelvic and para-aortic nodes and survived for 600 days before dying of disease. Analysis of survival and relapse-free survival is limited to the 48 patients who completed the planned dose of radiation.

Cisplatin

Cisplatin was given iv once weekly 2 hours before radiation. The first 4 patients were given a planned dose of 10 mg/m^2; the remaining patients received 20 mg/m^2. The infusion rate was 1 mg/minute. Antiemetic agents were administered im prior to the infusion, and iv hydration with mannitol was given 1 hour before the infusion to ensure a urine rate >75 ml/hour.

This dose level of cisplatin was chosen on the basis of the lack of toxicity observed with an identical regimen of cisplatin given concurrently with radiation in an Eastern Cooperative Oncology Group study (*17*) initiated at our institution for the treatment of patients with unresectable head and neck cancer.

Follow-up and Statistical Analysis

Median follow-up was 1.5 years from the start of treatment (range, 8 mo to 4 yr). Follow-up visits were every 1–2 months for the first year, every 3–4 months for the second and third years, and every 6 months for the fourth and fifth years.

Efficacy was evaluated using the end points of relapse-free survival and absolute survival. Actuarial curves were computed using the method of Kaplan and Meier.

RESULTS

Relapse-free Survival and Recurrence

The actuarial relapse-free survival for the entire group of treated patients was 43% at 2 and 3 years. In the subgroup of combined clinical stage I–III disease, relapse-free survival was 52% at 2 and 3 years.

Figure 1 gives relapse-free survival as a function of lymph node status and the presence or absence of peritoneal seeding. The subgroup of patients with involved para-aortic lymph nodes but without evidence of peritoneal seeding had relapse-free survival of 65% at 2 and 3 years. If all patients with involved para-aortic nodes, including those with peritoneal seeding, are taken as a group, the relapse-free survival decreases to 59% at 2 and 3 years.

The subgroup of patients with pelvic lymph node involvement only had 2- and 3-year relapse-free survival of 47%. The subgroup of patients with evidence of peritoneal seeding had relapse-free survival of 50% at 2 and 3 years. The majority of the subgroup with peritoneal seeding also had para-aortic node involvement.

Figure 2 gives relapse-free survival as a function of clinical stage.

In regard to recurrence, 47% of all recurrences occurred within 6 months, 78% within 12 months, and 100% by 2 years.

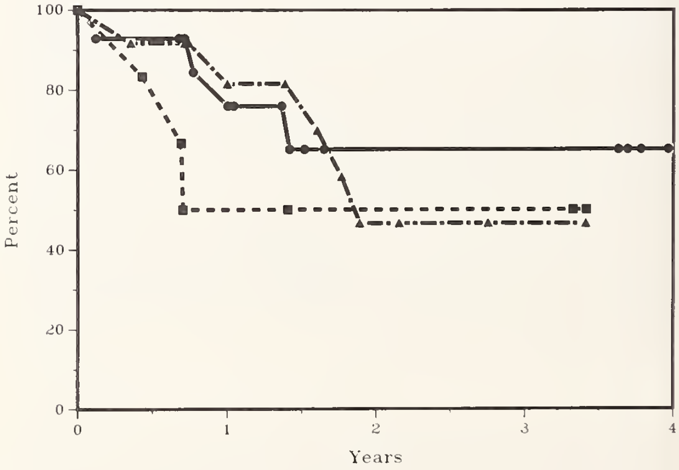


FIGURE 1.—Actuarial relapse-free survival rates vs. nodal status and presence or absence of peritoneal seeding. *Solid circle* = positive para-aortic lymph nodes with or without positive pelvic nodes; no peritoneal seeding. *Solid triangle* = pelvic lymph nodes only. *Solid square* = any peritoneal seeding.

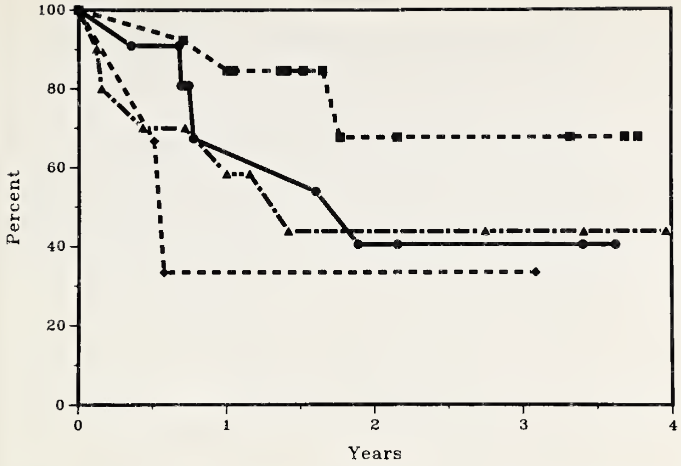


FIGURE 2.—Actuarial relapse-free survival rates vs. clinical stage. *Solid circle* = stage IB; *solid square* = stage II; *solid triangle* = stage III; *solid diamond* = stage IVA.

Absolute Survival

The absolute actuarial survival for the entire group of treated patients was 52% at 2 and 3 years. Survival for combined clinical stages I-III was 66% at 2 and 3 years.

The subgroup of patients with involved para-aortic lymph nodes but without evidence of peritoneal seeding had an excellent survival of 80% at 2 and 3 years. The subgroup of patients with pelvic lymph node involvement only had 2- and 3-year survival of 47%. Survival was 67% at 2 and 3 years for the subgroup with peritoneal seeding.

For the particular subpopulation of high-risk patients eligible for this study, there were no significant differences in survival at 2 and 3 years for clinical stages I-III. All patients with stage IVB disease died (median survival, 4.8 mo).

Acute Toxicity

No patient required reduction of the planned course of radiation because of acute toxicity. Median weight loss was 3.5 kg. This value compares favorably to historical values of 5.0 and 3.8 kg for patients previously treated without cisplatin who had extended-field radiation therapy with or without surgical staging, respectively (*1-3*).

In 6 (13%) of the patients, the nadir platelet count was $<$150,000 cells/mm^3, and in 4 (8%), the nadir platelet count was $<$100,000 cells/mm^3. No patient developed a nadir WBC count $<$2,000 cells/mm^3.

Chronic Toxicity

Three patients developed bowel obstruction requiring surgery, and 2 developed fistulas: 1 rectovaginal fistula in a patient with stage IVA cancer and 1 vesicovaginal fistula in a patient with a stage IIIB cancer. By comparison, in a historical control group of 41 patients treated at our institution with extended-field radiation and surgical staging, 4 women developed severe bowel injury (bowel obstruction or severe diarrhea requiring hospital admission); there were no fistulas (*2*).

One patient had a transient rise in BUN and creatinine that returned to normal. No additional nephrotoxicity has been noted.

DISCUSSION

Treatment of Advanced Cervical Cancer

Improving the prognosis of advanced cervical cancer will require both systemic treatment of occult disseminated disease and improvement in control of bulky local-regional disease. In a review of cervical cancer patients at Washington University, St. Louis, MO (*21*), the incidence of local-regional failure increased from 6% in clinical stage IB disease to 36% in stage III; distant failure increased from 8% in clinical stage IB to 40% in stage III. The high rates of distant metastases appear to be directly related to the presence or absence of pathologically involved pelvic or para-aortic nodes. At the University of Minnesota, in a study of patients with surgically staged cervical cancer with positive lymph nodes, 85% of the recurrences included distant failure (*2*). The 5-year survival decreased from 84% in patients with negative nodes to 50% and 40% in patients with pelvic and para-aortic node metastases, respectively.

One approach to these two problems is to find a chemotherapeutic agent with systemic efficacy to eradicate distant foci of disease and radiosensitizing effects to help improve local-regional control within the radiation field.

Improvement in prognosis using hydroxyurea as a radiation sensitizer in cervical cancer patients without para-aortic nodal metastases has been reported (*22-26*). However, adequate systemic efficacy was not demonstrated, and there was substantial toxicity.

It has been reported that cisplatin has modest activity in metastatic cervical cancer (*4-6*), with a response rate of 38% (*4*). Cisplatin also has radiosensitizing effects, which have been investigated both in animal models (*7-13*) and in clinical trials (*15-17*). Studies suggest that it is most effective to give cisplatin before radiation and that adequate tissue levels are obtained 2 hours after administration (*8,27*). These results formed the rationale for the design of the present clinical trial.

Toxicity

Radiation therapy alone is a treatment of proven efficacy in advanced cervical cancer, and it is important that any unproven adjunct treatment does not compromise its administration. No patient in the present trial required reduction in the planned dose of radiation because of the concurrent use of cisplatin. There were no measurable differences in acute toxicity from the use of adjuvant cisplatin except for the malaise, anorexia, nausea, and emesis on the day of the weekly cisplatin administration. Despite the use of extended radiation fields in a large number of patients, hematologic toxicity was not a significant problem.

Efficacy

Relapse-free survival in patients with pathologically confirmed nodal involvement who were treated with radiation alone has previously been reported from our institution. The 2- and 3-year relapse-free survival rates were 61% and 50%, respectively, for patients with pelvic lymph node involvement and 54% and 42%, respectively, for patients with para-aortic node involvement (*2*).

Comparison of these historical results with the results of

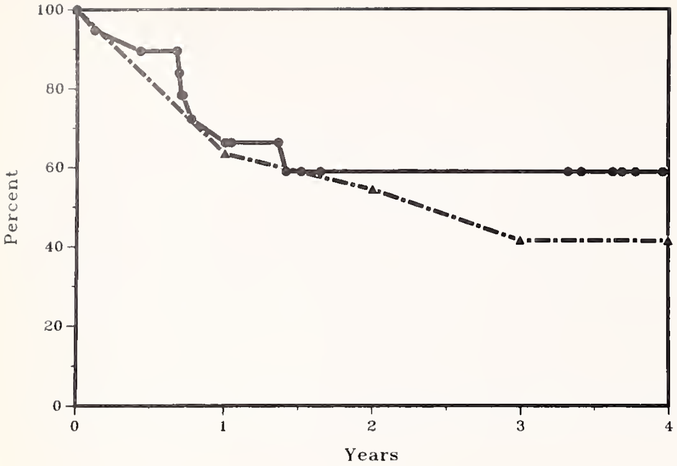


FIGURE 3.—Actuarial relapse-free survival rates in patients with positive para-aortic nodes: comparison between cisplatin-treated patients (*solid circle*) and historical controls (*solid triangle*).

the present trial is confounded by selection factors. Because of the entrance criteria for this trial, patients with pelvic node involvement were a prognostically poor subset of the entire population of patients with positive pelvic nodes. This probably accounts for the better relapse-free survival and absolute survival for patients with involvement of para-aortic nodes in this trial than for those with pelvic node involvement only. The better relapse-free survival in clinical stage II disease over stage I disease (fig. 2) is probably explained by the fact that only 6 (46%) of 13 patients with stage II disease were in the subgroup with relatively poor prognosis due to involvement of pelvic lymph nodes, whereas 8 (73%) of 11 patients with clinical stage I disease were in this group with relatively poor prognosis.

While only a subset of patients with positive pelvic nodes were entered in the present study, all patients with involved para-aortic nodes were eligible. Figure 3 shows the relapse-free survival for patients with involved para-aortic nodes in the present trial and in an otherwise unmatched historical control group from our institution (*2*). The curves for the first 1.5 years are nearly superimposed. After 1.5 years, there is gradual divergence of the two curves, with a suggestion of improved relapse-free survival for the cisplatin-treated group (59% vs. 54% at 2 yr and 59% vs. 42% at 3 yr).

The suggestion of this modest improvement in relapse-free survival for patients with positive para-aortic nodes and the need to identify other subsets of patients who may benefit lend support for the creation of a phase III prospective randomized trial to study the efficacy of adjuvant cisplatin and radiation in the treatment of advanced cervical cancer.

REFERENCES

(*1*) Potish R, Adcock L, Jones TK Jr, et al: The morbidity and utility of periaortic radiotherapy in cervical carcinoma. Gynecol Oncol 15:1-9, 1983.

(*2*) Potish RA, Twiggs LB, Okagaki T, et al: Therapeutic implications of the natural history of advanced cervical cancer as defined by pretreatment surgical staging. Cancer 56:956-961, 1985.

(*3*) Potish RA, Twiggs LB, Prem KA, et al: The impact of extraperitoneal surgical staging on morbidity and tumor recurrence following radiotherapy for cervical carcinoma. Am J Clin Oncol 7:245-251, 1984.

(*4*) Thigpen T, Shingleton H, Homesley H, et al: *cis*-Dichlorodiammineplatinum(II) in the treatment of gynecologic malignancies: Phase II trials by the Gynecologic Oncology Group. Cancer Treat Rep 63:1549-1555, 1979.

(*5*) Thigpen T, Shingleton H, Homesley H, et al: *Cis*-platinum in treatment of advanced or recurrent squamous cell carcinoma of the cervix: A phase II study of the Gynecologic Oncology Group. Cancer 48:899-903, 1981.

(*6*) Bonomi P, Bruchner MW, Cohen C: A randomized trial of three cisplatin regimens in squamous cell carcinoma of the cervix. Proc ASCO 1:110, 1982.

(*7*) Alvarez MV, Cobreros G, Heras A, et al: Studies on *cis*-dichlorodiammineplatinum(II) as a radiosensitizer. Br J Cancer 37:68-72, 1978.

(*8*) Szumiel I, Nias AHW: The effect of combined treatment with a platinum complex and ionizing radiation on Chinese hamster ovary cells in vitro. Br J Cancer 33:450-458, 1976.

(*9*) Chadwick KH, Leenhouts HP, Szumiel I, et al: An analysis of the interaction of a platinum complex and radiation with CHO cells using the molecular theory of cell survival. Int J Radiat Oncol Biol Phys 30:511-524, 1976.

(*10*) Richmond RC, Simic MG: Effect of radiation on *cis*-dichlorodiammineplatinum(II) and DNA in aqueous solution. Br J Cancer 37:20-23, 1978.

(*11*) Richmond RC, Zimbrick JD, Hykes DL: Radiation-induced DNA damage and lethality in *E. coli* as modified by the antitumor agent *cis*-dichlorodiammineplatinum(II). Radiat Res 71:447-460, 1977.

(*12*) Douple EB, Richmond RC: A review of platinum complex biochemistry suggests a rationale for combined platinum-radiotherapy. Int J Radiat Oncol Biol Phys 5:1335-1339, 1979.

(*13*) Yuhas JM, Tarleton AE, Culo F: Tumor line dependent interactions of irradiation and *cis*-diamminedichloroplatinum in the multicellular tumor spheroid system. Int J Radiat Oncol Biol Phys 6:1373-1375, 1979.

(*14*) Douple EB, Richmond RC: Platinum complexes as radiosensitizers of hypoxic mammalian cells. Br J Cancer 37:98-102, 1978.

(*15*) Reimer RR, Gahbauer R, Bukowski RM, et al: Simultaneous treatment with cisplatin and radiation therapy for advanced solid tumors: A pilot study. Cancer Treat Rep 65:219-222, 1981.

(*16*) Coughlin CT, Richmond RC: Platinum based combined modality approach for locally advanced head and neck carcinoma. Int J Radiat Oncol Biol Phys 11:915-919, 1985.

(*17*) Haselow RE, Adams GS, Oken MM, et al: Simultaneous *cis*-platinum (DDP) and radiation therapy (RT) for locally advanced unresectable head and neck cancer. Proc Am Assoc Cancer Res 24:624, 1983.

(*18*) Hoagland HC: Hematologic complications of cancer chemotherapy. Semin Oncol 9:95-102, 1982.

(*19*) Twiggs LB, Potish RA, McIntyre S, et al: Concurrent weekly *cis*-platinum and radiotherapy in advanced cervical cancer: Dose escalating toxicity study. Gynecol Oncol 24:143-148, 1986.

(*20*) Potish RA, Twiggs LB, Adcock LL, et al: Effect of *cis*-platinum on tolerance to radiation therapy in advanced cervical cancer. Am J Clin Oncol 9:387-391, 1986.

(*21*) Perez CA, Breaux S, Madoc-Jones H, et al: Radiation therapy alone in the treatment of carcinoma of the uterine cervix. I. Analysis of recurrence. Cancer 51:1393-1402, 1983.

(*22*) Hreschchyshyn MM, Aron BS, Boronow RC: Hydroxyurea of placebo combined with radiation to treat stage IIIB and IV cervical cancer confined to the pelvis. Int J Radiat Oncol Biol Phys 5:317, 1979.

(*23*) Piver MS, Barlow JJ, Vongtama V, et al: Hydroxyurea and radiation therapy in advanced cervical cancer. Am J Obstet Gynecol 120:969–972, 1974.

(*24*) Piver MS, Barlow JJ, Vongtama V, et al: Hydroxyurea as a radiation sensitizer in women with carcinoma of the uterine cervix. Am J Obstet Gynecol 129:379–383, 1977.

(*25*) Piver MS, Barlow JJ, Vongtama V: Hydroxyurea: A radiation potentiator in carcinoma of the uterine cervix. A randomized double blind study. Am J Obstet Gynecol 174:803–808, 1983.

(*26*) Piver MS, Krishnamsetty R, Emrich L: Survival of nonsurgically staged patients with negative lymphangiogram stage IIB carcinoma of the cervix treated by pelvic irradiation plus hydroxyurea. Am J Obstet Gynecol 151:1006–1008, 1985.

(*27*) Mattox DE, Sternson LA, Von Hoff DD, et al: Tumor concentration of platinum in patients with head and neck cancer. Otolaryngol Head Neck Surg 91:271–275, 1983.

Clinical Potential of Synchronous Radiotherapy and Chemotherapy for Advanced Squamous Cell Carcinoma[1]

Madhu J. John,* Marshall S. Flam, and Phyllis Ager Mowry[2,3]

ABSTRACT—**The equivocal results of past treatment regimens in which concurrent radiotherapy and chemotherapy were used may have resulted from a lack of site-specific active drugs that were also true radiation sensitizers. This report demonstrates an experience with 3 chemotherapeutic agents, 5-fluorouracil, cisplatin, and mitomycin, given simultaneously with radiation for locally advanced squamous cell carcinomas of such diverse sites as the anal canal, cervix, and esophagus. Early results show that the toxicity of these combination regimens is generally acceptable and indicate that such synchronous combination treatments may be superior in local control and survival to radiotherapy alone.—NCI Monogr 6:375-378, 1988.**

The first experiments in which CT was used in combination with RT were described by Kligerman and Shapiro (*1*), Bagshaw (*2*), and Kaplan et al. (*3*) in mammalian cells and tumor transplants. As early as 1961, Fletcher (*4*) showed increased oropharyngeal tumor regression with smaller total doses of RT when combined with FUra. In 1977, Phillips (*5*) categorized CT–RT interactions into the three classes of enhancement, interference, and antagonism. The antimetabolite FUra remains to this day the most frequently used drug in clinical studies aimed at modifying radiation response.

Knowledge of the advantages of FUra infusion over bolus injections and time sequence of RT–CT administration have been provided by Looney et al. (*6*) and Byfield and co-workers (*7*) among others. With the exception of RT with FUra and with CDDP administration, the RT–CT timing sequence has been mainly empirical. It has been based on the premise that CT drugs are incorporated into various points of the DNA molecular structure and make the tumor cell vulnerable to radiation damage by blocking sublethal repair.

Based on these new radiobiologic, pharmacologic, and clinical data, we used synchronous CT–RT regimens for advanced squamous cell carcinoma. This is an analysis of our treatment results in advanced cancers of the anal canal, esophagus, and cervix.

PATIENTS AND METHODS

Dose schedule.—The patients received 1,000 mg FUra · $m^2 \cdot day^{-1}$ by iv infusion for 4 days, 10–12 mg MMC/m^2 by iv bolus, and 75 mg CDDP/m^2 by iv infusion over 1–6 hours.

Anal canal.—Thirty consecutive patients with M_0 cloacogenic cancers were treated with a concurrent course of 3,600–4,500 cGy of pelvic RT and 2 courses of FUra–MMC between January 1979 and December 1985. In comparison to the original protocol of Wayne State University investigators (*8*) for this disease, 2 courses (instead of 1) of MMC were administered 3 weeks apart. Both CT courses were delivered during RT (instead of 1).

The RT field sizes and volume were adjusted to the bulk of disease, perineal skin involvement, and inguinal adenopathy. Further details of patient characteristics and techniques of RT and CT administration are described elsewhere (*9–11*). All patients were followed by digital and anoscopic examination, and computed axial tomography scans of the abdomen and pelvis; biopsies were taken when residual disease was suspected.

Esophagus.—Twenty-six consecutive patients with inoperable esophageal carcinoma were treated with a combined course of 5,040 cGy external RT and 4 courses of CT between December 1981 and March 1986. We delivered RT with a 2- to 3-cm margin beyond gross disease and excluded nodal drainage areas unless clinically involved. Alternating courses of FUra with MMC and CDDP were used, 3 courses concurrently with RT.

According to the American Joint Committee staging system (*12*), 5 patients had stage II disease, 14 had stage III, 4 had stage IV disease, and 3 patients had postsurgical recurrences. Patients were evaluated by endoscopy, biopsy, mediastinal computerized axial tomography scan, and esophagograms initially and at the conclusion of treatment.

Cervix.—From April 1983 to March 1986, all patients with advanced and nonmetastatic (M_0) squamous cell carcinoma of the cervix, FIGO stages IIB (poor prognosis), III, and IVA were offered concurrent CT and RT. Eleven patients accepted and are included in this analysis. A total of 3,600–4,500 cGy was delivered to the pelvis in 4 to 4½ weeks. Both FUra and MMC were given during the second week of RT. Two intracavitary applications with a Fletcher-Suit-Delclos application system followed, delivering a total of 4,000 mg-hours of radium-equivalent cesium. During the first intracavitary application, FUra and CDDP were administered.

ABBREVIATIONS: CT = chemotherapy; RT = radiation therapy; FUra = 5-fluorouracil; MMC = mitomycin; CDDP = cisplatin.

[1] Supported in part by Public Health Service grant CA-35283 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2] Departments of Radiation Oncology (*M. J. John* and *P. A. Mowry*) and Medicine (*M. S. Flam*), Fresno Community Hospital and Medical Center, CA, and the University of California, San Francisco.

[3] We thank Ms. Olivia Madrigal for assistance with this manuscript.

* *Reprint requests:* Madhu J. John, M.D., Department of Radiation Oncology, P.O. Box 1232, Fresno Community Hospital and Medical Center, Fresno, CA 93715.

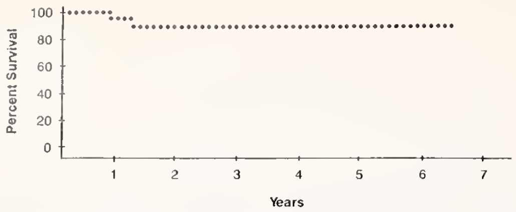


FIGURE 1.—Actuarial survival of patients with carcinoma of the anal canal.

All patients were staged and followed by pelvic examination and computed axial tomography scans of the abdomen and pelvis. The distribution of patients per FIGO classification were as follows: Stage IIB (poor prognosis): 3; III: 7; IVA: 1.

RESULTS

All patients were analyzed with regard to their disease status up to July 1986. These data were submitted for survival analysis by the actuarial life table method of Cutler and Ederer (*13*).

Anal Canal

The actuarial survival results of 30 patients with cancer of the anal canal are shown in figure 1. None of them died with disease, a fact not reflected in this unadjusted actuarial analysis. At the time of analysis, 1 patient had undergone anterior–posterior resection for recurrent disease 48 months after initiation of treatment.

Toxicity for this concurrent RT–CT regimen has been acceptable. All patients developed diarrhea and perineal dermatitis. Myelotoxicity was acceptable with nadir WBC counts of 2,250 and platelet counts of 95,000. There was one incident of anal fibrosis requiring dilatation in a patient who underwent an interstitial implant. No patient developed bowel toxicity requiring a colostomy.

Esophagus

Actuarial analysis of 26 patients showed a 60% survival rate at 1 year and a 27% survival rate at 2 years (fig. 2).

Even this aggressive regimen of 4 CT courses given concurrently with RT resulted in acceptable toxicity. There was 1 case each of sustained pancytopenia and thrombocytopenia, but nadir WBC and platelet counts of 2,250 and 110,000, respectively, were recorded. Esophageal fibrosis developed in 2 patients and was relieved in 1 with dilatation. There was 1 death secondary to pulmonary fibrosis in a patient with chronic obstructive pulmonary disease. Further details are available in a previous publication (*14*).

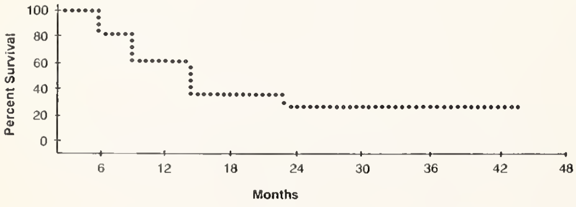


FIGURE 2.—Actuarial survival of patients with carcinoma of the esophagus.

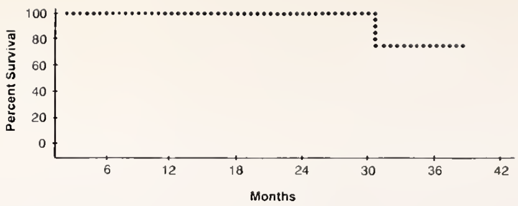


FIGURE 3.—Actuarial survival of patients with carcinoma of the cervix.

Cervix

Of 11 patients treated, none had evidence of disease at the end of our combined RT–CT protocol. One patient developed local and pulmonary disease at 21 months and died 7 months later. Another patient developed bone metastases at 22 months but remains alive with no other evidence of disease at this analysis (35 mo). The actuarial survival analysis is described in figure 3.

The major toxicity has been rectal ulcers in 2 patients that appear to be the result of intracavitary application and poor anatomy rather than RT–CT toxicity. One had salt depletion secondary to CDDP. Further toxicity details are available (*15*).

DISCUSSION

The sixties saw the advent of laboratory and clinical studies in which the regional and systemic modalities were combined. The most common agent administered was FUra, though 6-mercaptopurine, methotrexate, mechlorethamine, and cyclophosphamide were also used with less success. The doses of FUra varied and were delivered by weekly bolus injections, a technique we know now that is not conducive to maintenance of high serum levels required for "fixing" radiation-induced DNA damage (*7*).

Whereas one could always find a random study demonstrating increased tumor regression with RT–CT combinations, controlled studies invariably showed no improved survival with combined treatments. It may be noted that several researchers conducting these RT–CT studies used these modalities in sequential fashion, a trend that generally persists to this day.

The reports of Wayne State University researchers on combined treatment with FUra, MMC, and RT in anal canal cancers and especially in esophageal cancers gave impetus to a resurgence in interest in combined synchronous RT and CT (*8,16*). In 1983, Flam et al. (*9*) reported on 12 cases of anal canal cancers treated with combined RT and CT instead of surgery and first suggested radical surgery need not be done as a matter of course. A recent update on 30 patients reaffirms that original premise with long-term, disease-free status still being achieved without surgery (*11*).

In our series of inoperable esophageal cancers, we (*14*) reported a 57% complete remission rate and a 50% 1-year survival rate with an aggressive synchronous RT–CT regimen utilizing 5,040 cGy and 2 courses each of FUra with MMC and with CDDP. This differed from other reports, in that 3 drugs were administered for a total of 4 cycles in the Fresno Community Hospital and Medical Center series compared with 2 drugs for 2 cycles in others (*14,16*). The

TABLE 1.—Results of synchronous radiotherapy and chemotherapy for squamous cancers of various sites

Site	No. of patients	Alive with/without disease	Dead with/without disease	Follow-up, mo (median)
Anal canal	30	0/27	0/3	8–77 (38)
Esophagus	26	1/10	13/2	1–44 (8)
Cervix	11	1/9	1/0	3–38 (17)

results in median survival and 1-year survival rates were superior with this aggressive approach and were without increased toxicity (*14*).

Our experience with advanced and poor prognostic-featured cervical cancers continues to hold with a 100% initial complete clinical response at the end of the combined regimen using 3 drugs and slightly reduced doses of radiotherapy. Our results with these 3 tumor sites are summarized in table 1.

The interaction between infusional FUra and RT appears to be that of enhancement or potentiation rather than an additive effect (*17*). Both Looney et al. (*6*) and Byfield and associates (*7*) have shown that maximal tumor cell kill occurs when FUra is administered by continuous infusion during and following RT. Both CDDP and MMC have been shown to be effective single agents in squamous cell carcinoma. Both are alkylating radiomimetic agents in that they cause cross-linking of DNA and consequent chromosomal damage. Their simultaneous use with and without radiation is impeded by apparent synergism in myelotoxic effect (*18*). The effectiveness of their individual concomitant use with FUra, however, has been established in several clinical trials with esophageal and anal cancers (*8,16*). Similarly, the pharmacokinetics of their interaction with RT has been described (*19,20*).

We have used alternating courses of FUra–MMC and FUra–CDDP to reduce toxicities of the 2 alkylating agents. Currently available clinical and laboratory data demonstrate that these 3 drugs cause radiation enhancement and improved tumor response. Toxicity analyses of this treatment series and others suggest that the therapeutic ratio is increased.

In these advanced tumors with significant tumor bulk, when it is obvious that neither RT nor CT given alone would cause sufficient cell kill, it is possible that the *sequential* administration of RT and CT could allow repair of sublethal damage and redistribution to relatively insensitive cell phases. Inversely, the *concurrent* use of RT–CT could decrease such repair and possibly promote synchronization and differential cell kill. Whereas some drugs (e.g., FUra) may increase tumor cell kill at the primary site by enhancement alone, others (CDDP or MMC) in addition may eradicate tumor deposits at distant sites or circulating tumor cells and thereby decrease the incidence of distant metastases (*21*).

Our experience suggests that concomitant RT–CT given in proper doses and sequences have been very effective in the initial treatment of advanced squamous cancers (anal canal, esophagus, and cervix). Further experience with this approach with newer radiobiologic and pharmacokinetic data should have an important effect on the management of many solid tumors.

REFERENCES

(*1*) KLIGERMAN MM, SHAPIRO DM: Augmentation of radiotherapeutic effect of cancer chemotherapy. Radiology 69:194–200, 1957.

(*2*) BAGSHAW MA: Possible role of potentiators in radiation therapy. Am J Roentgenol 85:822–833, 1961.

(*3*) KAPLAN HS, SMITH KC, TOMLIN PA: Radiosensitization of *E. coli* by urine and pyrimidine analog incorporated in deoxyribonucleic acid. Nature 190:794–796, 1961.

(*4*) FLETCHER GH: Some observations of combined therapy with 5-fluorouracil and radiation, with special reference to oropharyngeal carcinoma. *In* Research in Radiotherapy (Kallman RF, ed). Nuclear Science Ser, Rep No. 35. Washington, DC: Natl Acad Sci Press, 1961, pp 210–218.

(*5*) PHILLIPS TL: Chemical modification of radiation effects. Cancer 39:987–999, 1977.

(*6*) LOONEY WB, HOPKINS HA, MACLEOD MS, et al: Solid tumor models for the assessment of different treatment modalities. XII. Combined chemotherapy-radiotherapy: Variation of time interval between time of administration of 5-fluorouracil and radiation and its effect on the control of tumor growth. Cancer 44:437–445, 1979.

(*7*) BYFIELD JE, CALABRO-JONES P, KLISAK I, et al: Pharmacologic requirements for obtaining sensitization of human tumor cells in vitro to combined 5-fluorouracil or ftorafur and X rays. Int J Radiat Oncol Biol Phys 8:1923–1933, 1982.

(*8*) NIGRO ND, VAITKEVICIUS VK, CONSIDINE B: Combined therapy for cancer of the anal canal. A preliminary report. Dis Colon Rectum 17:354–356, 1974.

(*9*) FLAM MS, JOHN MJ, LOVALVO L, et al: Definitive nonsurgical therapy of epithelial malignancies of the anal canal. A report of 12 cases. Cancer 51:1378–1387, 1983.

(*10*) JOHN MJ, FLAM M, LOVALVO L, et al: Feasibility of non-surgical definitive management of anal canal carcinoma. Int J Radiat Oncol Biol Phys 13:299–303, 1987.

(*11*) FLAM MS, JOHN M, MOWRY P, et al: Definitive combined modality therapy of carcinoma of the anus: A report of 30 cases including results of salvage therapy in patients with residual disease. Dis Colon Rectum 30:495–502, 1987.

(*12*) BEAHRS OH, MYERS MH (eds): Manual for Staging of Cancer, 2nd ed. Philadelphia: Lippincott, 1983, pp 61–64.

(*13*) CUTLER SJ, EDERER F: Maximum utilization of the life-table method of analysis of survival. J Chronic Dis 8:699–713, 1958.

(*14*) JOHN MJ, FLAM M, WITTLINGER P, et al: Inoperable esophageal carcinoma: Results of aggressive synchronous radiotherapy and chemotherapy: A pilot study. Am J Clin Oncol 10:310–316, 1987.

(*15*) JOHN M, COOKE JK, FLAM M, et al: Preliminary results of concomitant radiotherapy and chemotherapy in advanced cervical carcinoma. Gynecol Oncol 28:101–110, 1987.

(*16*) LEICHMAN L, STEIGER Z, SEYDEL HG, et al: Combined preoperative chemotherapy and radiation therapy for cancer of the esophagus: The Wayne State University, Southwest Oncology Group and Radiation Therapy Oncology Group experience. Semin Oncol 11:178–185, 1984.

(*17*) MANDEL HG: Incorporation of 5-fluorouracil with RNA and its molecular consequences. Prog Mol Subcell Biol 1:82–135, 1969.

(*18*) STOLL DB, LAVIN PT, ENGSTROM PF: Hematologic toxicity of cisplatin and mitomycin in combination for squamous cell carcinoma of the esophagus. Am J Clin Oncol 8:231–234, 1985.

(*19*) ROCKWELL S, KENNEDY K: Combination therapy with radiation and mitomycin C. Preliminary results with EMT6 tumor cells in vitro and in vivo. Int J Radiat Oncol Biol Phys 5:1673–1676, 1979.

(*20*) Kyriazis AP, Yagoda A, Kereiakes JG, et al: Experimental studies on the radiation-modifying effect of *cis*-diamminedichloroplatinum II (DDP) in human bladder transitional cell carcinomas grown in nude mice. Cancer 52:452–457, 1983.

(*21*) John M, Flam M, Wittlinger P, et al: Clinical evidence of feasibility and efficacy of simultaneous irradiation and chemotherapy in advanced squamous cell carcinoma. *In* Advances in Cancer Control: Health Care Financing and Research. New York: Alan R Liss, 1986, pp 287–301.

Discussion Summary

Timothy J. Kinsella[1] **and Evan B. Douple**[2,*]

PROTOCOL DESIGN FOR COMBINING CISPLATIN WITH RADIATION THERAPY

It is apparent from the posters discussed in this session that cisplatin has become a popular drug choice for the design of combined modality protocols that incorporate a chemotherapy agent with radiation. Whether this has resulted primarily from a rationale based upon preclinical radiobiology studies or from the broad spectrum activity that cisplatin has demonstrated in the medical oncology field is not clear. The tumors discussed in clinical protocols are principally those known to have some response to cisplatin, such as head and neck, esophageal, anal canal, bladder, and cervix cancers. The tumors treated with the combined modality approach also include small cell and non-small cell lung carcinomas and osteosarcomas, tumors in which cisplatin has shown some limited activity. However, published preclinical results demonstrating interactions between platinum compounds and radiation, plus the promise for less toxic second-generation platinum agents, have also helped promote this class of metallo-organic compounds as attractive modifiers of radiation response.

Although the posters and presentations of the participants specified cisplatin doses, including a wide range from 20 to 100 mg/m^2, delivered according to a variety of schedules, the precise intervals between the administration of the platinum and radiation were frequently omitted. The reports of most of the phase I studies at this meeting described the scheduling in such ambiguous terms as "concurrent," "the day of," "concomitant," "just prior to," or "simultaneous." It was stressed during the discussion that if mechanisms of radiation potentiation are to be exploited, precise scheduling of each of the combined modality treatments will probably be required. However, several participants pointed out the constraints that optimum timing might place on the radiation and medical oncology communities. It is clear that coordination of the two modalities will require careful communication and cooperation between the two disciplines.

Several of the authors presenting protocols indicated that they would like to know the concentrations and active species of platinum compound or adduct that would most likely be required to produce a significant interaction with radiation and to improve the therapeutic index with respect to a particular tumor type or individual patient. Whereas reports of pharmacokinetic studies have included mention of steady-state or peak plasma levels of platinum, it was suggested that intracellular levels of platinum or platinum-DNA adducts in tumors might be more relevant parameters for predicting enhancement of radiation damage. It was also proposed that the use of rapidly developing technologies, such as monoclonal antibodies to platinum-DNA adducts, might better define the role of platinum in patients receiving the combined therapies. Until such information is available, protocol design will continue to be heavily influenced by practical considerations, such as management of emesis, patient scheduling, and diagnosis-related group constraints, thus producing phase I trials that are more likely to test whether 1) strictly additive effects of the two modalities are improving tumor responses, and 2) the effects on normal tissues are dose limiting or well tolerated, rather than to investigate whether an interaction between cisplatin and radiation is exploitable or optimized. One of the basic concerns was that we simply do not know whether a single large bolus dose of cisplatin coordinated with a few doses of radiation is better than multiple low doses of cisplatin throughout the treatment schedule. Other factors such as circadian rhythm might play an important role in determining relevant platinum distributions.

EXACERBATION OF NORMAL TISSUE DAMAGE

Although results of several studies claimed a demonstration of protocol feasibility, some participants reported evidence of enhancement of acute normal toxicity in the combined modality trials, especially in the small intestine and lung as could have been predicted from the published results of certain preclinical studies in animal systems. Most authors suggested that these toxicities are manageable, but others were concerned that we need to have more information about late toxicities. Some evidence for apparent enhancement of radiation-induced pneumonitis and pulmonary fibrosis was presented. It is difficult in a phase I study for an investigator to determine whether an apparent increase in morbidity relative to historical experience is significant and is a direct result of the combined modality protocol.

CLINICAL EVALUATION OF TUMOR RESPONSE

The participants were concerned that phase I–II studies will not be able to provide answers to the important questions regarding the role of drug dose and timing in improving tumor control. In small trials, tolerance to treatment and regimen feasibility are being assessed in selected and heterogeneous patient populations. It will probably require at least two stages of clinical experimentation to advance this field. The first stage will include phase III randomized trials with control arms to demonstrate that in a particular tumor type the combination is going to lead to improved efficacy. A second stage of phase III trials will then be needed to refine dosage, timing, and sequencing details. There is always the risk that because the protocols of studies in the first stage of testing are not optimized they will fail to demonstrate a positive effect. It is hoped that the latter stage phase III studies will be based upon preclinical radiobiologic information from ongoing experimentation as well as the results of the first stage of ongoing phase III trials.

---

[1] Radiation Oncology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[2] Radiobiology Laboratories, Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Hanover, NH.

[*] *Reprint requests:* Evan B. Douple, Ph.D., Radiobiology Laboratories, Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Hanover, NH 03756.

The concern was raised that modest dose modification factors for radiation therapy applied to only a minor percentage of the total treatments might not produce a significant overall effect. It was pointed out that a demonstrably improved therapy could result simply from the additive effects of the 2 agents. Randomized, cooperative group phase III trials ongoing in both the United States and in Europe may resolve some of these uncertainties, but it is too early for the investigators of these studies to report the results. Scientists who presented reports at this meeting are claiming improved tumor response rates, but it is not clear whether these responses will necessarily lead to improved cure rates with acceptable morbidity.

The end point of "histologic complete response" as a monitor of tumor response was discussed. It was stressed that the histologic complete response cannot assure that there is no tumor in the treatment field and also that we do not know what the meaning of such a response may be for long-term survival. Finally, someone mentioned that in many of the protocols other chemotherapeutic agents were used. For example, head and neck cancer trials frequently include 5-fluorouracil, an agent that has been reported to interact with radiation. Earlier clinical trials have reported therapeutic enhancement following combinations of 5-fluorouracil and radiation. Unless phase III trials have appropriate control arms, it will be impossible for us to ascertain whether the enhanced effect upon the tumor or normal tissues is due to the cisplatin or, at least in part, to other chemotherapeutic agents.

Priority Announcement Service Available for Future Issues

If you would like to be notified when additional *NCI Monographs* are issued, please complete the form below and mail to the address on the form. (You will also be notified when other cancer-related publications are issued.)

MAIL TO: Superintendent of Documents, US Government Printing Office, Washington, DC 20402

Please add my name to your Priority Announcement List (N-569) to be notified when new *NCI Monographs* are issued.

Company or Personal Name

Additional address/attention line

Street address

City State ZIP Code

(or Country)

PLEASE PRINT OR TYPE

MAIL TO: Superintendent of Documents, US Government Printing Office, Washington, DC 20402
Make checks payable to Superintendent of Documents.

Please send me the following volume(s) of *Cancer Treatment Symposia*:

____ copies of the Proceedings of the Workshop on Patterns of Failure After Cancer Treatment (Volume 2, 1983), Stock No. 017-042-00167-6 ($15.00 per copy domestic, $18.75 foreign).

____ copies of The Interdisciplinary Program for Radiation Oncology Research (Volume 1, 1984), Stock No. 017-042-00171-4 ($6.50 per copy domestic, $8.15 foreign).

____ copies of the Proceedings of the Conference on 2′-Deoxycoformycin: Current Status and Future Directions (Volume 2, 1984), Stock No. 017-042-00172-2 ($5.00 per copy domestic, $6.25 foreign).

____ copies of the Proceedings of the Workshop on Mitoxantrone Cardiotoxicity and the Workshop on Clinical Evaluation of Anthracycline Cardiotoxicity (Volume 3, 1984), Stock No. 017-042-00180-3 ($4.75 per copy domestic, $5.95 foreign).

____ copies of the Proceedings of the Third Annual Sanrocco International Meeting on Clinical and Biological Evaluation of the Immunomodifiers (Volume 1, 1985), Stock No. 017-042-00181-1 ($5.50 per copy domestic, $6.90 foreign).

____ copies of the Proceedings of the Workshop on Radiotherapy for Lung Cancer (Volume 2, 1985), Stock No. 017-042-00183-8 ($8.50 per copy domestic, $10.65 foreign).

____ copies of the Proceedings of the National Institutes of Health Consensus Development Conference on Limb-Sparing Treatment of Adult Soft Tissue Sarcomas and Osteosarcomas (Volume 3, 1985), Stock No. 017-042-00185-4 ($6.50 per copy domestic, $8.15 foreign).

____ copies of Compilation of Phase II Results With Single Antineoplastic Agents (Volume 4, 1985), Stock No. 017-042-00189-7 ($22.00 per copy domestic, $27.50 foreign).

Enclosed is $________ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐-☐

Order No. ____________

MasterCard and VISA accepted.

Credit Card Orders Only
Total charges $________
Fill in the boxes below

Credit Card No. ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date Month/Year ☐☐☐☐

Customer's Telephone No.'s

Area Code	Home	Area Code	Office

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8:00 a.m. to 4:00 p.m. Eastern Time Monday-Friday (except holidays)

Company or Personal Name
☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Additional address/attention line
☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Street address
☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

City | State | ZIP Code
☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

(or Country)
☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

PLEASE PRINT OR TYPE

For Office Use Only

Quantity		Charges
______	Publications	______
______	Subscriptions	______
	Special Shipping Charges	______
	International Handling	______
	Special Charges	______
	OPNR	______
______	UPNS	
______	Balance Due	
______	Discount	
______	Refund	

NCI Monographs in the JNCI Series

Volumes Available

No. 57: Surveillance, Epidemiology, and End Results Program: Incidence and Mortality Data: 1973–77
#017-042-00156-1; $26.00 (U.S.), $32.50 (elsewhere)

No. 59: Cancer Mortality in the United States: 1950–1977
#017-042-00161-7; $18.00 (U.S.), $22.50 (elsewhere)

No. 61: Third International Symposium: Cancer Therapy by Hyperthermia, Drugs, and Radiation
#017-042-00160-9; $21.00 (U.S.), $26.25 (elsewhere)

No. 64: Management Operations of the National Cancer Institute That Influence the Governance of Science
#017-042-00175-7; $9.00 (U.S.), $11.25 (elsewhere)

No. 65: Use of Small Fish Species in Carcinogenicity Testing
#017-042-00173-1; $16.00 (U.S.), $20.00 (elsewhere)

No. 66: Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens
#017-042-00176-5; $12.00 (U.S.), $15.00 (elsewhere)

No. 67: Selection, Follow-up, and Analysis of Prospective Studies: A Workshop
#017-042-00182-0; $14.00 (U.S.), $17.50 (elsewhere)

No. 68: Multiple Primary Cancers in Connecticut and Denmark
#017-042-00186-2; $23.00 (U.S.), $28.75 (elsewhere)

No. 69: Fourth Symposium on Epidemiology and Cancer Registries in the Pacific Basin
#017-042-00188-9; $16.00 (U.S.), $20.00 (elsewhere)

No. 70: Forty-five Years of Cancer Incidence in Connecticut: 1935–79
#017-042-00187-1; $37.00 (U.S.), $46.25 (elsewhere)

To: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402

Please send me ________ copies of Monograph No. ________, (title) ________________________________

____

Stock # ________________________; Price ______________

Make checks payable to Superintendent of Documents.

Enclosed is $______ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐ - ☐

Order No. __________

MasterCard and VISA accepted.

Credit Card Orders Only
Total charges $______
Fill in the boxes below

Customer's Telephone No
Area Code | Home | Area Code | Office

Credit Card No. ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date Month/Year ☐☐☐☐

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8:00 a.m. to 4:00 p.m. Eastern Time: Monday–Friday (except holidays).

Company or Personal Name

Additional address/attention line

Street address

City
State
ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity		Charges
______	Publications	______
	Special Shipping Charges	______
	Special Charges	______
	OPNR	______
______	UPNS	
______	Balance Due	
______	Discount	
______	Refund	

GPO

JNCI Monographs

The International Cancer Information Center of the National Cancer Institute announces publication of the first issue of the new *NCI Monographs:* Proceedings of the NIH Consensus Development Conference on Adjuvant Chemotherapy and Endocrine Therapy for Breast Cancer.

During the past decade, systemic therapy has been added to local-regional treatment of breast cancer to prevent recurrence and improve survival of patients. This volume of *NCI Monographs* presents data from many scientists and clinical investigators, who participated in an NIH consensus development conference that was held September 9–11, 1985. Based on these presentations, important conclusions were drawn with respect to improvements in relapse-free survival, appropriate patients for therapy, and the toxic effects of these treatments. Taken together, these data represent one of the most significant advances in the treatment of human solid tumors and should be of interest to all involved in the management of patients with cancer.

Copies of this volume of *NCI Monographs* (Number 1, 1986) are available from the U.S. Government Printing Office ($8.50 per copy domestic, $10.65 foreign). To order, request Stock No. 017-042-00190-1. Make checks payable to the Superintendent of Documents and mail to: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

To: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402

Please send me ________ copies of Monograph No. ________, (title) ______________________________

____

Stock # ______________________________; Price ____________________

Make checks payable to Superintendent of Documents.

Enclosed is $________ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐-☐

Order No. ____________

MasterCard and VISA accepted.

Credit Card Orders Only
Total charges $______
Fill in the boxes below

Customer's Telephone No
Area Code Home
Area Code Office

Credit Card No.

Expiration Date
Month/Year

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8:00 a.m. to 4:00 p.m. Eastern Time: Monday–Friday (except holidays)

Company or Personal Name

Additional address/attention line

Street address

City
State
ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity		Charges
______	Publications	______
	Special Shipping Charges	______
	Special Charges	______
	QPNR	______
______	UPNS	
______	Balance Due	
______	Discount	
______	Refund	

GPO

NCI Monographs

"Cancer Control Objectives for the Nation: 1985–2000" is the second of the new *NCI Monographs* to be published by the International Cancer Information Center of the National Cancer Institute.

This report is the result of the work and collaboration of four Working Groups composed of experts from the United States, Canada, and the staff of the National Cancer Institute. By setting specific objectives, with quantified indicators for measuring progress, and by recommending actions for achieving these objectives, the Working Groups present in this monograph a consensus statement on the potential progress toward cancer control that can be attained by the American public with the help of the concerted efforts of public and private agencies. The goal of a marked reduction in the cancer mortality rate by the year 2000 is a unifying theme of the current cancer control program.

To order copies of this volume (Number 2, 1986) from the United States Government Printing Office, request Stock No. 017-042-00191-9 and enclose payment ($6.00 per copy domestic, $7.50 foreign). Please make checks payable to Superintendent of Documents and mail to: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

To: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402

Please send me ________ copies of Monograph No. ________, (title) ________________

Stock # ________________; Price ____________

Make checks payable to Superintendent of Documents.

Enclosed is $______ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐ - ☐

Order No. ____________

MasterCard and VISA accepted.

Credit Card Orders Only
Total charges $______
Fill in the boxes below

Customer's Telephone No
Area Code — Home — Area Code — Office

Credit Card No. ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date
Month/Year ☐☐☐☐

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8:00 a.m. to 4:00 p.m. Eastern Time, Monday–Friday (except holidays).

Company or Personal Name

Additional address/attention line

Street address

City — State — ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity		Charges
______	Publications	______
	Special Shipping Charges	______
	Special Charges	______
	OPNR	______
______	UPNS	
______	Balance Due	
______	Discount	
______	Refund	

GPO

NCI Monographs

The International Cancer Information Center of the National Cancer Institute announces publication of the third issue of the new *NCI Monographs:* International Symposium on Labeled and Unlabeled Antibody in Cancer Diagnosis and Therapy (Number 3, 1987).

This volume of *NCI Monographs* contains the proceedings of a symposium held at The Johns Hopkins Hospital (Baltimore, MD), which focused on the use of antibodies in cancer diagnosis and treatment. The meeting represents the beginning of dialogue among investigators who have done research in the disciplines of physics, radiobiology, immunology, and pharmacology and those who have clinical expertise in the fields of nuclear medicine and radiation oncology. The work reported at this meeting involves use of dosimetry, low-dose-rate radiation, and antibody fragments, as well as pilot studies of isotope linkage, chemical cytotoxic antibodies, and diagnostic and therapeutic programs. These presentations demonstrate the promise of antibody therapy, which can deliver a broad range of cytotoxic agents, often without the acute side effects of other types of therapy.

Copies of this volume of *NCI Monographs* (Number 3, 1987) are available from the U.S. Government Printing Office ($9.50 per copy domestic, $11.90 foreign). To order, request Stock No. 017-042-00194-3. Make checks payable to the Superintendent of Documents and mail to: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

To: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402

Please send me ________ copies of Monograph No. ________, (title) ______________________________

____

Stock # ______________________________; Price ________________

Make checks payable to Superintendent of Documents.

Enclosed is $________ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐ - ☐

Order No. ____________

MasterCard and
VISA accepted.

Credit Card Orders Only
Total charges $________
Fill in the boxes below

Customer's Telephone No
Area Code | Home | Area Code | Office

Credit Card No ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date Month/Year ☐☐☐☐

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8:00 a.m. to 4:00 p.m. Eastern Time: Monday–Friday (except holidays).

Company or Personal Name

Additional address/attention line

Street address

City State ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity		Charges
______	Publications	______
	Special Shipping Charges	______
	Special Charges	______
	OPNR	______
______	UPNS	
______	Balance Due	
______	Discount	
______	Refund	

GPO

NCI Monographs

The proceedings of the meeting on "Development of Folates and Folic Acid Antagonists in Cancer Chemotherapy" have been published in *NCI Monographs* by the International Cancer Information Center of the National Cancer Institute.

In 1986, the National Cancer Institute sponsored a meeting of leading investigators who are studying the use of folic acid antagonists in cancer chemotherapy. It has been known for many years that compounds that interfere with folic acid metabolism inhibit the proliferation of cancer cells. Several new antifols have now been developed, and participants at this meeting discussed advances in preclinical and clinical knowledge and the most desirable directions for future research.

Copies of this volume of *NCI Monographs* (Number 5, 1987) are available from the U.S. Government Printing Office ($12.00 per copy domestic, $15.00 foreign). To order, request Stock No. 017-042-00215-0. Make checks payable to the Superintendent of Documents and mail to: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

To: Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402

Please send me ________ copies of Monograph No. ________, (title) ____________________

Stock # ________________; Price ________

Make checks payable to Superintendent of Documents.

Enclosed is $______ ☐ check,
☐ money order, or charge to my
Deposit Account No.

☐☐☐☐☐☐☐ - ☐

Order No. ________

MasterCard and VISA accepted.

Credit Card Orders Only
Total charges $______
Fill in the boxes below

Customer's Telephone No
Area Code Home Area Code Office

Credit Card No. ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date Month/Year ☐☐☐☐

Charge orders may be telephoned to the GPO order desk at (202)783-3238 from 8:00 a.m. to 4:00 p.m. Eastern Time: Monday–Friday (except holidays)

Company or Personal Name

Additional address/attention line

Street address

City State ZIP Code

(or Country)

PLEASE PRINT OR TYPE

For Office Use Only

Quantity		Charges
______	Publications	______
	Special Shipping Charges	______
	Special Charges	______
	OPNR	______
______	UPNS	
______	Balance Due	
______	Discount	GPO
______	Refund	

NCI Monographs

ONCOLOGY OVERVIEWS

The International Cancer Information Center of the National Cancer Institute announces publication of the following ONCOLOGY OVERVIEWS. ONCOLOGY OVERVIEWS are specialized bibliographies with abstracts, each referencing 150–500 recent publications on a cancer topic of high current interest, drawn from over 3000 sources. Leading researchers in the field covered by each OVERVIEW review and select the most relevant and significant abstracts for each topic, resulting in a tightly focused, quick reference to the most recent cancer literature.

DIAGNOSIS AND THERAPY SERIES

Bone Marrow Transplantation in Cancer,
Stock No. 017-042-00201-0, $5.50

VIROLOGY, IMMUNOLOGY AND BIOLOGY SERIES

Viral Etiology of Cancer II. Papillomaviruses,
Stock No. 017-042-00199-4, $5.50

Viral Etiology of Cancer III. Hepatitis B Virus,
Stock No. 017-042-00197-8, $6.50

Idiotypes, Anti-Idiotypes and Network Regulation,
Stock No. 017-042-00202-8, $6.00

Transforming Growth Factors and Other Autocrine Growth Factors,
Stock No. 017-042-00200-1, $5.00

CARCINOGENESIS SERIES

Translocation and Amplification of Oncogenes,
Stock No. 017-042-00198-6, $6.00

Oncogene Protein Products,
Stock No. 017-042-00205-2, $5.50

Chemopreventive Agents,
Stock No. 017-042-00204-4, $6.50

Potential Occupational Causes of Cancer II. The Urinary Tract,
Stock No. 017-042-00203-6, $4.75

Dioxins and Dibenzofurans in Carcinogenesis, 1980–1986,
Stock No. 017-042-00206-1, $5.50

Radioprotectors: Experimental Studies and Clinical Applications,
Stock No. 017-042-00207-9, $6.50

Superintendent of Documents Publication Order Form

Order Processing Code:
* 6284

Charge your order. It's easy!

1. **Please Type or Print** (Form is aligned for typewriter use.)

All prices include regular domestic postage and handling and are good through 3/88. After this date, please call Order and Information Desk at 202-783-3238 to verify prices.

Qty.	Stock Number	Title	Price Each	Total Price
		International customers please add an additional 25%	Total for Publications	

Please Type or Print

2. ____________________
(Company or personal name)

____
(Additional address/attention line)

____
(Street address)

____
(City, State, ZIP Code)

(   ) ____________________
(Daytime phone including area code)

3. **Please Choose Method of Payment:**

☐ Check payable to the Superintendent of Documents

☐ GPO Deposit Account ☐☐☐☐☐☐☐–☐

☐ VISA, CHOICE or MasterCard Account

☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

____
(Credit card expiration date)

Thank you for your order!

____
(Signature)

4. **Mail To:** Superintendent of Documents, Government Printing Office, Washington, D.C. 20402-9325

5/87

225 Inhibition of Radiation-induced DNA–Protein Cross-link Repair by Glutathione Depletion With L-Buthionine Sulfoximine

231 Radioprotection of Rat Subependymal Plate With 4-OH Sodium Butyrate

235 WR-2721 as Cytotoxic and Radioprotective Agent in Treatment of Murine Lymphoma With Total Body Irradiation

241 Discussion Summary

Combined Modality Treatment—I

247 Keynote Address: Combined Modality Treatment for Primary Tumor

253 An Organized Multi-institutional Interdisciplinary Evaluation of Role of Radiation Therapy Alone or Combined With Chemotherapy in Treatment of Adenocarcinoma of the Gastrointestinal Tract

259 Phase III Trial of Irradiation Plus Chemotherapy for Patients With Hepatic Metastases and Hepatoma: Experience of the Northern California Oncology Group

265 Patterns of Failure in Combined Chemotherapy and Radiotherapy for Limited Small Cell Lung Cancer: Southeastern Cancer Study Group Experience

271 Use of Hemibody Irradiation as a Non-cross-resistant Agent in Combination With Systematic Chemotherapy in Small Cell Lung Cancer

275 Ten-year Results of Randomized Trial Comparing Radiotherapy and Concomitant Bleomycin to Radiotherapy Alone in Epidermoid Carcinomas of the Oropharynx: Experience of the European Organization for Research and Treatment of Cancer

279 Combined Modality Approach to Treatment of Malignant Gliomas—Re-evaluation of RTOG 7401/ECOG 1374 With Long-term Follow-up: A Joint Study of the Radiation Therapy Oncology Group and the Eastern Cooperative Oncology Group

285 Pilot Study of Interaction of Radiation Therapy With Doxorubicin by Continuous Infusion

291 Treatment of High-risk Sarcomas in Children and Young Adults: Analysis of Local Control Using Intensive Combined Modality Therapy

297 Integration of Full-dose Adjuvant Chemotherapy With Definitive Radiotherapy for Primary Breast Cancer: Four-year Update

303 Comparison of Total Nodal Irradiation Versus Combined Sequence of Mantle Irradiation With Mechlorethamine, Vincristine, Procarbazine, and Prednisone in Clinical Stages I and II Hodgkin's Disease: Experience of the European Organization for Research and Treatment of Cancer

311 Discussion Summary

Combined Modality Treatment—II

315 Keynote Address: Platinum–Radiation Interactions

321 Combined Modality Treatment of Peritoneal Mesotheliomas

323 Continuous Infusion of Etoposide, Bolus Administration of Cisplatin, and Simultaneous Radiation Therapy in Previously Treated Patients With Small Cell Bronchogenic Carcinoma

327 Concomitant Therapy With Infusion of Cisplatin and 5-Fluorouracil Plus Radiation in Stage III Non-Small Cell Lung Cancer

331 Phase II Trial of Therapy With Etoposide, 5-Fluorouracil by Continuous Infusion, Cisplatin, and Simultaneous Split-course Radiation in Stage III Non-Small Cell Bronchogenic Carcinoma

335 Combination of Chemotherapy and Radiotherapy in Limited Small Cell Lung Carcinoma: Results of Alternating Schedule in 109 Patients

339 Simultaneous Therapy With High-dose Cisplatin and Radiation for Unresectable Squamous Cell Cancer of the Head and Neck: A Phase I-II Study

343 Concomitant Therapy With Infusion of Cisplatin and 5-Fluorouracil Plus Radiation in Head and Neck Cancer

347 Simultaneous Radiotherapy and Chemotherapy With 5-Fluorouracil and Cisplatin for Locally Confined Squamous Cell Head and Neck Cancer

353 Sequential Administration of Methotrexate, Cisplatin, and 5-Fluorouracil in Multimodal Therapy for Locally Advanced Head and Neck Cancer

357 Chemotherapy With Low Doses of Radiation Followed by Definitive Radiotherapy for Advanced Unresectable Carcinoma of the Head and Neck

361 New Strategies for Avoiding Total Laryngectomy in Patients With Head and Neck Cancer

365 Clinical Experience With High-dose Cisplatin and Radiation for Treatment of Epithelial Malignancies

369 Tolerance and Preliminary Results of Simultaneous Therapy With Radiation and Cisplatin for Advanced Cervical Cancer

375 Clinical Potential of Synchronous Radiotherapy and Chemotherapy for Advanced Squamous Cell Carcinoma

379 Discussion Summary

NCI Monographs

NIH LIBRARY
3 1496 00340 5084

A0851
ACQUISITIONS UNIT - JOURNALS
NIH LIBRARY
BUILDING 10, ROOM 1-L-13

1 Introduction

Late Toxic Effects in Normal Tissues

5 Keynote Address: Models of Normal Tissue Injury Following Combined Modality Therapy

9 Special Lecture: Scoring of Late Toxic Effects—Interaction of Two Modalities

19 Interaction of Cisplatin and X-rays in Rat Kidney

23 Renal Damage in Mice After Treatment With Cisplatin and X-rays: Comparison of Fractionated and Single-dose Studies

29 Effect of Total-body Irradiation With Bone Marrow Transplantation on Toxicity of Cisplatin

35 Effect of Sequencing on Combined Toxicity of Renal Irradiation and Cisplatin

41 DNA Damage and Repair in Patients Receiving High-dose Cyclophosphamide and Radiation

45 Long-term Consequences of Chemotherapeutic Agents on Hematopoiesis: Development of Altered Radiation Tolerance

51 Modification of Cyclophosphamide-induced Pulmonary Toxicity in Normal Mice

55 Effect of Continuous Infusion of Bleomycin on Repopulation in Mouse Lip Mucosa During Fractionated Irradiation

59 Rodent Model of Chemoradiotherapy-induced White Matter Necrosis

65 Quantitative Risk of Second Cancer in Patients in First Complete Remission From Early Stages of Hodgkin's Disease

73 Discussion Summary

Mechanisms of Interaction Resistance—I

77 Keynote Address: Mechanisms of Interaction Between Radiation and Drugs With Potential for Improvements in Therapy

85 Special Lecture: Alternating Chemotherapy and Radiotherapy

95 Response of Cell Subpopulations in Spheroids to Radiation-Drug Combinations

101 Responses of Tumor Cell Subpopulations to Single Modality and Combined Modality Therapies

107 Increase in Hypoxic Fraction of Human Colon Tumor Xenografts by Preirradiation of Tumor Bed

111 Development and Application of a Rat Tumor Model for Human Bronchial Carcinoma

115 Effect of Spirogermanium and Radiation Therapy on the 9L Rat Brain Tumor Model

119 Potentiation of Rat Brain Tumor Therapy by Fluosol and Carbogen

123 Plasma and Tumor Concentrations of Cisplatin Following Intraperitoneal Infusion or Bolus Injection With or Without Continuous Low-dose-rate Irradiation

129 Platinum Levels in Murine Tumor Following Intraperitoneal Administration of Cisplatin or Paraplatin

133 Improvement of Differential Toxicity Between Tumor and Normal Tissues Using Intratumoral Injection With or Without a Slow-drug-release Matrix System

137 Response of Murine Tumors to Matrix-associated Cisplatin Intratumoral Implants

141 Effects of Matrix-associated Chemotherapy in Combination With Irradiation In Vivo

145 Administration of Radiation or Cyclophosphamide Versus Alternated Radiation and Cyclophosphamide Treatment of Primary Tumor and Pulmonary Metastases

147 Systematic Investigation Into Interaction of Ionizing Radiation and Doxorubicin in the Dunning R3327G Prostatic Adenocarcinoma Model

155 Discussion Summary

Mechanisms of Interaction Resistance—II

159 Keynote Address: Mechanisms of Cross-resistance Between Radiation and Antineoplastic Drugs

167 Inhibition of Radiation Dose-rate-sparing Effects in Human Tumor Cells by 3-Aminobenzamide

173 Effects of Methylxanthines on Cell-cycle Redistribution and Sensitization to Killing by Low-dose-rate Radiation

177 Interactions Between Antitumor Drugs and Radiation in Mammalian Tumor Cell Lines: Differential Drug Responses and Mechanisms of Resistance Following Fractionated X-Irradiation or Continuous Drug Exposure In Vitro

183 Cell-cycle, Phase-specific Cell Killing by Carmustine in Sensitive and Resistant Cells

187 Chinese Hamster Pleiotropic Multidrug-resistant Cells Are Not Radioresistant

193 Altered Radiosensitivity of Hematopoietic Stem Cells by Vincristine Pretreatment: Superoxide Dismutase Activity as a Possible Mechanism

199 Inhibition of Pentose Cycle of A549 Cells by 6-Aminonicotinamide: Consequences for Aerobic and Hypoxic Radiation Response and for Radiosensitizer Action

205 Radioresistance of Human Tumor Xenografts: Possible Mechanisms

211 Glutathione as a Determinant of Cellular Response to Doxorubicin

217 Cell-cycle Dependence of X-ray Oxygen Effect: Role of Endogenous Glutathione

NIH Publication No. 88-2939 Number 6 1988

THE NIH LIBRARY
NATIONAL INSTITUTES OF HEALTH

LIBRARY
NIH
Amazing Research.
Amazing Help.
http://nihlibrary.nih.gov

10 Center Drive
Bethesda, MD 20892-1150
301-496-1080

INFORMATION
LB
CONSERVATION, INC

[illegible] 1998

NIH LIBRARY
3 1496 00380 6737